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<p>(21) International Application Number: PCT/US98/10645 (22) International Filing Date: 20 May 1998 (20.05.98) (30) Priority Data: 60/047,162 20 May 1997 (20.05.97) US 60/053,652 24 July 1997 (24.07.97) US 08/991,840 16 December 1997 (16.12.97) US (71) Applicant (for all designated States except US): WALTER REED ARMY INSTITUTE OF RESEARCH [-/US]; Dept. of the Army, Washington, DC 20307 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): PARKER, Michael, D. [US/US]; Apartment T-13, 1001 Carroll Parkway, Frederick, MD 21701 (US). SMITH, Jonathan, F. [US/US]; 6936 Eyler Valley Flint Road, Sabillasville, MD 21780 (US). CRISE, Bruce, J. [US/US]; 7210 McKaig Road, Frederick, MD 21701 (US). OBERSTE, Mark, Steve [US/US]; 5110 Sunset Maple Trace, Lilburn, GA 30247 (US). SCHMURA, Shannon, M. [US/US]; 10812 Lincoln Avenue, Hagerstown, MD 21740 (US).</p>	<p>(74) Agent: HARRIS, Charles, H.; United States Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: LIVE ATTENUATED VIRUS VACCINES FOR EQUINE ENCEPHALITIS VIRUSES</p>		
<p>(57) Abstract cDNAs coding for an infectious Western Equine Encephalitis virus (WEE) and infectious Venezuelan Equine Encephalitis virus variant IE (VEE IE) are disclosed in addition to cDNA coding for the structural proteins of Venezuelan Equine Encephalitis virus variant IIIA (VEE IIIA). Novel attenuating mutations of WEE and VEE IE and their uses are described. Also disclosed are attenuated chimeric alphaviruses and their uses.</p>		

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TITLE OF THE INVENTION

LIVE ATTENUATED VIRUS VACCINES FOR EQUINE ENCEPHALITIS VIRUSES

5

10

INTRODUCTION

Western equine encephalitis (WEE), eastern equine
15 encephalitis (EEE) and Venezuelan equine encephalitis
virus (VEE) are members of the alphavirus genus of the
family Togaviridae which is comprised of a large group
of mosquito-borne RNA viruses found throughout much of
the world. The viruses normally circulate among
20 rodent or avian hosts through the feeding activities
of a variety of mosquitoes. Epizootics occur largely
as a result of increased mosquito activity after
periods of increased rainfall. Western equine
encephalitis virus (WEE) was first recognized in 1930
25 and causes periodic outbreaks of disease in equines.
The virus has been detected over much of the western
hemisphere from Argentina north to the more temperate
regions of central Canada (For a review, see Reisen
and Monath [1988] in The Arboviruses: Epidemiology and
30 Ecology, Vol. V. CRC Press, Inc. Boca Raton).

Similarly, EEE was first isolated in Virginia and New
Jersey in 1933 (Ten Broeck, C. et al. [1935] *J. Exp.*
Med. 62:677) and is now known to be focally endemic
throughout much of the northern portion of South

America, Central America and the eastern part of Mexico and the United States. Venezuelan equine encephalitis virus has six serological subtypes (I-VI). Two of these subtypes, I and III have multiple variants, two of these variants are of particular interest in this application, variant IE, and variant IIIA also called Mucambo virus. A live, attenuated vaccine (TC-83) for VEE IA/B has been used for immunization equines and at-risk laboratory and field personnel (Birge et al. [1961] *Am. J. Hyg.* 73:209-218; Pittman et al. [1996] *Vaccine* 14:337-343). The vaccine was credited with helping to limit the northward spread of a serious epizootic of VEE originating in South America in the late 1960's. However, the VEE I/AB vaccines have not yet been licensed by the Food and Drug Administration and have been shown to be effective in preventing disease from VEE IA/B infection only. The current VEE vaccines do not adequately protect against the VEE IE variant or the VEE IIIA variant, as disease has occurred in laboratory workers successfully vaccinated with a vaccine derived from VEE IA/B. In addition, recent unprecedented outbreaks of VEE IE in populations of horses in Mexico indicate a need for a VEE IE vaccine. The lack of adequate cross protection with existing IA/B vaccines documents the need for a VEE IE-specific and a VEE IIIA-specific vaccine.

The vaccines currently in veterinary use for WEE, EEE and VEE IA/B throughout the United States and Canada are formalin-inactivated preparations. Inactivated vaccines for EEE and WEE are also available for use by at-risk laboratory personnel. These inactivated vaccines are poorly immunogenic, require multiple inoculations with frequent boosters and generally result in immunity of short duration.

The shortcomings of the available vaccines indicate a need for the development of new vaccines of high immunogenicity which induce a longer lasting immunity for protection against WEE, EEE and VEE subtypes IE and IIIA.

SUMMARY OF THE INVENTION

The present invention satisfies the need mentioned above.

10 In this application are described live attenuated vaccines for WEE, EEE, VEE IE and VEE IIIA which may provide higher level immunity in humans and equines for many years, and possibly for life. In addition, very large numbers of vaccine doses can be produced
15 from significantly less starting materials than is possible with the existing inactivated products. The vaccine preparations of the present invention comprise full-length cDNA copies of the genomes of WEE or VEE IE which have been altered such that the RNA produced
20 from the cDNA, and the virus produced therefrom is attenuated and useful as a live vaccine for human and veterinary use. The vaccine preparations for VEE IIIA and EEE are novel chimeric viruses which include the newly discovered structural protein genes of VEE IIIA.

25 The classic methods of deriving live-attenuated vaccines (blind passage in cell cultures) generally result in heterogeneous and undefined products, hence recent attempts to make live vaccines for alphaviruses have relied on genetic engineering procedures.

30 The alphavirus genome is a single-stranded, positive-stranded RNA approximately 11,400 nucleotides in length. The 5' two-thirds of the genome consist of a non-coding region of approximately 48 nucleotides followed by a single open reading frame of
35 approximately 7,500 nucleotides which encodes the

viral replicase/transcriptase. The 3' one-third of the genome encodes the viral structural proteins in the order C-E3-E2-6K-E1, each of which are derived by proteolytic cleavage of the product of a single open reading frame of approximately 3700 nucleotides. The sequences encoding the structural proteins are transcribed as a 26S mRNA from an internal promoter on the negative sense complement of the viral genome. The nucleocapsid (C) protein possesses autoproteolytic activity which cleaves the C protein from the precursor protein soon after the ribosome transits the junction between the C and E3 protein coding sequence. Subsequently, the envelope glycoproteins E2 and E1 are derived by proteolytic cleavage in association with intracellular membranes and form heterodimers. E2 initially appears in the infected cell as a precursor, pE2, which consists of E3 and E2. After extensive glycosylation and transit through the endoplasmic reticulum and the golgi apparatus, E3 is cleaved from E2 by furin-like protease activity at a cleavage site having a consensus sequence of RX(K/R)R, with X being one of many amino acids present in the different viruses, and with the cleavage occurring after the last arginine residue. Subsequently, the E2/E1 complex is transported to the cell surface where it is incorporated into virus budding from the plasma membrane (Strauss and Strauss [1994] *Microbiological Rev.* 58: 491-562). All documents cited herein *supra* and *infra* are hereby incorporated in their entirety by reference thereto.

Because the genome of alphavirus is a positive-stranded RNA, and infectious upon transfection of cells in culture, an "infectious clone" approach to vaccine development is particularly suitable for the alphaviruses. In this approach, a full-length cDNA

clone of the viral genome is constructed downstream from a RNA polymerase promoter, such that RNA which is equivalent to the viral genome can be transcribed from the DNA clone *in vitro*. This allows site-directed
5 mutagenesis procedures to be used to insert specific mutations into the DNA clone, which are then reflected in the virus which is recovered by transfection of the RNA.

Previous work with infectious clones of other
10 alphaviruses has demonstrated that disruption of the furin cleavage site results in a virus which incorporates pE2 into the mature virus. Davis *et al.* (1995, *supra*) found that disruption of the furin cleavage site in an infectious clone of VEE is a
15 lethal mutation. Transfection of BHK cells with RNA transcribed from this mutant clone resulted in the release of non-infectious particles. However, a low level of infectious virus was produced which contained secondary suppressor mutations such that virus
20 containing pE2 was fully replication competent and subsequently shown to be avirulent but capable of eliciting immunity to lethal virus challenge in a variety of animal species.

The genetic basis for attenuation of the VEE TC-
25 83 vaccine and certain laboratory strains of VEE virus have been studied extensively and has led to the development of improved live, attenuated vaccine candidates (Kinney *et al.* 1993, *supra*, Davis *et al.* 1995, *supra*). The approach used in this application
30 is similar to that used for VEE, however, following the VEE example exactly did not result in an adequate vaccine for WEE. Changes in the procedure used for VEE were required, none of which could have been predicted from the VEE work, in order to produce the
35 attenuated live WEE virus of the present invention.

Based upon a comparison of the structural protein gene sequences of WEE and other alphaviruses, the probable furin cleavage site of WEE strain CBA/87 virus is RRPKR. The presence of the extra arginine when compared to the consensus (RX(R/K)R) alphavirus cleavage site indicated that the cleavage at this site might be more complex than that observed with VEE virus. It was necessary therefore to prepare two deletion mutations in the E3-E2 cleavage site of the full-length clone, one which lacks five amino acids and one which lacks four amino acids since it was unknown which mutation, if any, would produce an attenuated virus. The residual arginine in the full-length clone lacking only four amino acids was of concern due to the possibility that other mutations might arise due to the presence of the extra arginine resulting in cleavage by cellular proteases at that site and producing an apparently wild type virus with respect to cleavage of pE2.

Transfection of cultured cells with RNA transcribed from an infectious clone of WEE lacking the furin cleavage site yielded viruses which contained the pE2 of WEE in the mature virus but which were not replication competent. During intracellular replication of the RNA, mutations arise at low frequency, resulting in a small number of replication competent virus. Sequence analysis of these viruses has shown that the lethal effect of the deletion mutations was alleviated by the appearance of second site mutations in the E2 glycoprotein. These viruses are attenuated in mice when administered by subcutaneous or intracranial inoculation. The mice produce high titer neutralizing and ELISA antibody and are protected against a lethal challenge of parental virulent WEE virus.

Therefore, in one aspect of the invention, the invention pertains to the isolation of a cDNA sequence coding for an infectious western equine encephalitis (WEE) virus RNA transcript. DNA representing the entire genome, not previously available, was prepared by polymerase chain reaction using a series of primer pairs based upon the partial genome sequences previously deposited in Genbank. The 5' and 3' ends of the viral genome were unknown and difficult to obtain. The terminal sequence was necessary for efficient replication of the virus since substitution of ends from a similar virus with similar but not identical sequences resulted in an extremely attenuated virus. In order to determine the correct sequence at the 5' end, a protocol called rapid amplification of cDNA ends (5'-RACE) was used. The full length infectious clone is useful in the production of virulent WEE virus, and introducing and testing attenuating mutations. The production of virulent virus is essential for a formal measure of the degree of attenuation achieved with candidate attenuating mutations and a formal determination of the rate at which reversion to virulence might occur.

In another aspect of the invention, the invention pertains to the isolation of a cDNA sequence coding for an infectious Venezuelan equine encephalitis virus IE variant (VEE IE) virus RNA transcript (SEQ ID NO: 2). Using oligonucleotides specific to genomic RNA of a VEE IE isolate (GenBank accession no. U34999) (Oberste, et al. [1996] *Virology* 219:314-320), reverse transcriptase polymerase chain reaction was carried out to generate numerous cDNA fragments which were subsequently cloned and used to assemble full-length cDNA of VEE IE. The full length infectious clone is useful, for example, in the production of

virulent VEE IE virus, and introducing and testing attenuating mutations.

In the case of VEE IIIA, the structural protein genes were removed from a full length clone of VEE IA/B and replaced by the VEE IIIA structural genes. The IIIA structural gene sequences were prepared by RT-PCR and include the IIIA 26S promoter and the 3' nontranslated region (3'NTR) flanking the sequences for the structural proteins (SEQ ID NO:3). The VEE IIIA 3' NTR was then replaced with the VEE IA/B NTR, and the modified sequence was cloned back into the VEE IA/B full length clone. The result was a clone in which the nonstructural protein gene sequences were from VEE IA/B and the structural protein gene sequences from VEE IIIA. The virus produced from this chimeric clone replicated efficiently in cell culture, and proved to be completely attenuated in mice. In addition, it was highly immunogenic and protected the vaccinated mice against challenge with virulent, wild-type Mucambo virus (VEE IIIA).

Portions of the cDNA sequences described above are useful as probes to diagnose the presence of virus in samples, and to define naturally occurring variants of the virus. These cDNAs also make available polypeptide sequences of WEE antigens, EEE antigens, VEE IE antigens, and VEE IIIA structural polypeptide antigens encoded within the respective genomes and permits the production of polypeptides which are useful as standards or reagents in diagnostic tests and/or as components of vaccines. Antibodies, both polyclonal and monoclonal, directed against WEE epitopes, EEE epitopes, VEE IE epitopes, or VEE IIIA epitopes contained within these polypeptide sequences are also useful for diagnostic tests, as therapeutic agents, and for screening of antiviral agents.

Accordingly, with respect to polynucleotides, some aspects of the invention are: a purified WEE polynucleotide; a recombinant WEE polynucleotide; a recombinant polynucleotide comprising a sequence
5 derived from a WEE genome or from WEE cDNA; a recombinant polynucleotide encoding an epitope of WEE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transfected with any of these vectors.

10 Other aspects of the invention are: a purified VEE IE polynucleotide; a recombinant VEE IE polynucleotide; a recombinant polynucleotide comprising a sequence derived from a VEE IE genome or from VEE IE cDNA; a recombinant polynucleotide
15 encoding an epitope of VEE IE; a recombinant vector containing any of the above recombinant polynucleotides, and a host cell transformed with any of these vectors.

In a further aspect of the invention is a
20 complete sequence of the VEE subtype IIIA structural protein genes useful for diagnostics and vaccine development. Also provided is a chimeric virus containing the structural sequences of VEE IIIA, which is completely attenuated and provides protection
25 against challenge with VEE IIIA virulent virus for use as a vaccine

Another aspect of the invention is a single-stranded DNA sequence comprising a cDNA clone coding for an infectious WEE, the cDNA clone including at
30 least one attenuating mutation therein, the RNA produced from transcription of the cDNA and the virus particles produced from the RNA in a host cell for use as a vaccine.

In another aspect of the invention there is
35 provided a full length WEE cDNA clone containing a

defined deletion mutation useful for attenuating the virus for the identification of suppressor mutations in the virus. The attenuated virus with the cleavage deletion and suppressor mutations are useful as a
5 means to generate an attenuated, live WEE virus vaccine for veterinary and human use.

In a further aspect of the invention is provided a chimeric virus containing nonstructural protein gene sequences from WEE and structural protein gene
10 sequences from any alphaviruses including but not limited to Aura, Barmah Forest, Bebaru, Cabassou, Chikungunya, eastern equine encephalitis, Everglades, Fort Morgan, Getah, Highlands J, Kyzylgach, Mayaro, Middelburg, Mucambo, Ndumu, O'nyong-nyong, Pixuna,
15 Ross River, Sagiyama, Semliki Forest, SAAR87, Sindbis, Tonate, Una, Venezuelan equine encephalitis, Whataroa, which could be used as a means for attenuating virulent alphaviruses, and vaccine production against other alphaviruses.

20 Taking advantage of the close evolutionary relationship between WEE and eastern equine encephalitis virus (EEE), a chimeric virus has been constructed in which the structural protein genes of EEE have been inserted into the infectious clone in
25 place of the WEE structural protein genes. The resulting virus is fully replication competent, attenuated, and elicits an immune response in mice which is protective against a lethal challenge with virulent EEE virus.

30 In addition, depending on the non-WEE sequences substituted for the WEE structural genes, another aspect of the invention includes a means for expressing antigens of other alphaviruses as chimeric alphaviruses for use as potential vaccines for human
35 and veterinary use.

In another aspect of the invention there is provided a full length infectious VEE IE cDNA clone containing a cleavage deletion useful in the identification of suppressor mutations in the virus,
5 the RNA produced from the cDNA and the virus produced from the RNA. The virus with the cleavage deletion and suppressor mutations is useful as a means to generate an attenuated, live VEE IE vaccine virus for veterinary and human use.

10 In a further aspect of the invention is provided a chimeric virus containing nonstructural sequences from VEE IE and structural sequences from other alphaviruses which could be used as a means of attenuating virulent alphaviruses.

15 In addition, depending on the non-VEE IE sequences substituted for the structural sequences of VEE IE, another aspect of the invention includes a means to express antigens of other alphaviruses as chimeric alphaviruses as potential vaccines for human
20 and veterinary use.

In a further aspect of the invention, there is provided a vaccine protective against WEE, the vaccine comprising live attenuated WEE virus in an amount effective to elicit protective antibodies in an animal
25 to WEE and a pharmaceutically acceptable diluent, carrier, or excipient.

In yet a further aspect of the invention, there is provided a vaccine protective against VEE IE, the vaccine comprising live attenuated VEE IE virus in an
30 amount effective to elicit protective antibodies in an animal to VEE IE and a pharmaceutically acceptable diluent, carrier, or excipient.

In another aspect of the invention, there is provided a bivalent vaccine protective against WEE and
35 VEE IE, the vaccine comprising both attenuated WEE and

attenuated VEE IE in an amount effective to elicit protective antibodies in an animal to both WEE and VEE IE and a pharmaceutically acceptable diluent. In addition, it is possible that this vaccine will
5 provide short lived protection against other alphaviruses (Cole and McKinney [1971] *Inf. Immunity* 4:37-43).

In yet another aspect of the invention, there is provided an inactivated vaccine produced from the live
10 attenuated virus described above. The attenuated virus of the present invention whether whole virus or chimeric virus can be used in producing inactivated virus vaccines. By using an attenuated virus strain, there is a much greater margin of safety in the event
15 that the produce is incompletely inactivated. Starting with an attenuated strain is also much safer during the manufacturing phase, and allows production under lower biocontainment levels.

20 BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

25
Figure 1A, 1B and 1C. Assembly of the full length cDNA clone of western equine encephalitis virus. Polymerase chain reaction products representing the entire genome of WEE virus were
30 prepared with the primer pairs indicated. Each of the products was cloned into pBluescript KS+. Assembly of the full length clone, pWE2000 in pBluescript was carried out as indicated in the figure and described below. The clones are not drawn to scale. In each of
35 the plasmids, the primers used to generate the PCR

products are indicated as are the restriction endonuclease sites used for the assembly.

Figure 2. Polypeptide profiles of western equine encephalitis viruses. Samples of purified virus were analyzed by electrophoresis on 10% polyacrylamide gels and stained with Comassie Brilliant blue. Molecular weight marker (MW) are shown in the first lane and molecular weights are indicated in daltons X 10⁻³. The virus strain is designated above the appropriate lane. Position of contaminating bovine serum albumin is indicated by arrow.

Figure 3. Polypeptide profiles of western equine encephalitis virus (WEE), eastern equine encephalitis (EEE) and chimeric virus, MWE. Samples of purified virus were analyzed by electrophoresis on 10% polyacrylamide gels and stained with Comassie Brilliant blue. Molecular weight markers (MW) are shown and molecular weights are indicated in daltons X 10⁻³. Position of contaminating bovine serum albumin is indicated by arrow.

Figure 4. Derivation of virulent VEE IE clone. Constructions of cDNA encoding the entire genome of cloned strain 68U201 are shown. The relevant cloning sites and genetic markers are indicated above each clone. The first full-length clone produced that replicated *in vitro* after transfection of the RNA transcribed from the T7 promoter, was pIE-1006. Further modifications to this clone were carried out in order to obtain pIE-1009 that had the *in vitro* and *in vivo* characteristics of the parental, biological isolate, strain 68U201. With a fully virulent clone, pIE-1009 available, specific attenuating mutations

were introduced into pIE-1009 to generate the attenuated clone, pIE1100. Drawings are not to scale. Shaded areas represent regions of pIE1006 that were replaced or mutated to generate new clones.

5

Figure 5. Construction of IAB-IIIA cDNA chimeric clone pV3A-1000.

DETAILED DESCRIPTION

10 In one embodiment, the present invention relates to a full length cDNA clone of fully virulent WEE virus specified in SEQ ID NO:1, and a full length cDNA clone of fully virulent VEE IE variant specified in SEQ ID NO:2.

15 WEE, strain CBA/87, isolated from the brains of an infected horse in Argentina in 1987 (Bianchi et al., [1987] *Am.J. Trop. Med. Hyg.* 49:322-328) was used as a parent strain in the instant invention. Any other strain which consistently kills 100% of 5 week old
20 C57BL6 mice when inoculated subcutaneously can be used such as B11, for example. The cDNA clone can be generated by any of a variety of standard methods known in the art. Preferably, DNA representing the entire genome can be prepared by polymerase chain
25 reaction using a series of primer pairs based upon the partial genome sequences previously deposited in GenBank. The 5' terminal sequence of the virus may be determined by 5'-RACE basically as described by Frohman et al. ([1988] *Proc. Natl. Acad. Sci. U.S.A.*
30 85:8998-9002). Assembly of the full length clone can be in a suitable transcription vector such as, for example, pBluescript KS+, using convenient restriction endonuclease sites or the entire genome could be inserted into any plasmid which contains suitable
35 restriction endonuclease cleavage sites for cloning,

an origin of replication so that the plasmid can be propagated in a bacterial host, and a selectable marker gene to maintain the plasmid in the bacterial cell during growth. The DNA sequence preferably has a complementary DNA sequence bonded thereto so that the double-stranded sequence will serve as an active template for RNA polymerase. Hence, the transcription vector preferably comprises a plasmid. When the DNA sequence comprises a plasmid, it is preferred that a unique restriction site be provided 3' (with respect to the virion RNA sequence) to ("down-stream" from) the cDNA clone. This provides a means for linearizing the DNA sequence to enhance the efficiency of transcription of genome-length RNA *in vitro*.

The complete clone is preferably operatively associated with a promoter region such that the promoter causes the cDNA clone to be transcribed in the presence of an RNA polymerase which binds to the promoter. The promoter is positioned on the 5' end (with respect to the virion RNA sequence), or upstream from, the cDNA clone. An excessive number of nucleotides between the promoter sequence and the cDNA clone will result in the inoperability of the construct. Hence, the number of nucleotides between the promoter sequence and the cDNA clone is preferably not more than eight, more preferably not more than than 5, still more preferably not more than 3, and most preferably not more than 1. Exemplary promoters useful in the present invention include, but are not limited to, T3 promoters, T7 promoters, and SP6 promoters. It is preferable that the 5' end of the *in vitro* transcript not have any additional nucleotides preceding the first nucleotide of the viral sequence. At the 3' end, additional nucleotides can be tolerated in the *in vitro* transcript but are probably lost when

the virus replicates. In most instances, the poly-dA tract at the 3' end is required for viability of the virus. Selection of the virulent full length clone can be achieved by comparing the LD₅₀ of the virus encoded by the cloned cDNA with the LD₅₀ of the parent virus used, in the instant example, WEE CBA/87. The ability to produce virulent virus is important; it allows the introduction and testing of attenuation mutations and the attenuated phenotype against a standard: the virulent parent.

Transfection of cells with the RNA transcript coded by the full length genomic cDNA can be achieved by any suitable means, such as, for example, by treating the cells with DEAE dextran, treating the cells with "LIPOFECTIN", and by electroporation. Togavirus-permissive cells, alphavirus-permissive cells, and WEE-permissive and VEE IE-permissive cells are cells which, upon transfection with the viral RNA transcript, are capable of producing viral particles. Togaviruses have a broad host range. Examples of such cells include, but are not limited to, Vero cells, baby hamster kidney cells, chicken embryo fibroblast cells, Chinese hamster ovary cells (CHO), mouse L cells, MRC-5 cells, mosquito cells such as C6-36 cells, to name a few.

In the case of VEE IE, an isolate of VEE IE, strain 68U201, was used. Any isolate known to cause disease in man can be chosen. In addition the ability of a strain to have an easily identifiable phenotype such as, for example, the ability to form large plaques in tissue culture on indicator cell monolayers, is helpful. In the present invention, the full length clone of VEE IE was obtained using oligonucleotide primers specific for the VEE IE strain 68U201 sequence. Reverse transcription-polymerase

chain reaction (RT-PCR) of strain 68U201 viral RNA was carried out to generate numerous cDNA fragments that were subsequently cloned and used to assemble a full-length cDNA in a plasmid such that the cDNA could be
5 precisely transcribed and viral infectious RNA produced. It is also possible to produce the entire genome by polymerase chain reaction by including the promoter sequence in the 5' end primer thereby producing infectious RNA directly from the PCR
10 fragment. It is also possible to transcribe the viral RNA from a plasmid in the cell by transfection with the appropriate plasmid containing a promoter utilized by cellular RNA polymerases, i.e. the CMV promoter.

To determine virulence of the cloned viral
15 genome, mice can be inoculated subcutaneously with 10^4 plaque forming units of the cloned virus. The clone is considered virulent if all mice die, and not fully virulent if mice do not all die. The LD_{50} of parent VEE IE strain 68U201 is to 1-2 pfu, therefore,
20 if inoculation of 10,000-fold did not cause lethal disease in all mice, it was considered attenuated.

In another embodiment of the present invention is provided a cDNA sequence of the entire 26S region of VEE subtype IIIA as well as the structural protein
25 genes specified in SEQ ID NO:3.

DNA or polynucleotide sequences to which the invention also relates include sequences of at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12
30 nucleotides, most preferably at least about 15-20 nucleotides corresponding, i.e., homologous to or complementary to, a region of the WEE, VEE IE, or VEE IIIA nucleotide sequence. Preferably, the sequence of the region from which the polynucleotide is derived is
35 homologous to or complementary to a sequence which is

unique to the virus. Whether or not a sequence is unique to the virus can be determined by techniques known to those of skill in the art. For example, the sequence can be compared to sequences in databanks, e.g., GenBank. Regions from which typical DNA sequences may be derived include but are not limited to, for example, regions encoding specific epitopes, as well as non-translated regions.

The derived polynucleotide is not necessarily physically derived from the nucleotide sequence of the alphaviruses, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain reaction assays and for the discovery of other alphavirus sequences.

A polypeptide or amino acid sequence derived from the amino acid sequence of alphavirus, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with as a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid

sequence, it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system.

Once a complete viral genomic cDNA is cloned,
5 attenuation of the virus is possible. An attenuating mutation refers to a nucleotide mutation or amino acid coded for in view of such a mutation which results in a decreased probability of causing disease in its host (i.e., a loss of virulence), in accordance with
10 standard terminology in the art. The attenuating mutation may be a substitution mutation or an in-frame deletion mutation.

Novel WEE attenuating mutations disclosed herein which may be used to carry out the present invention
15 include deletion of five amino acids at the furin cleavage site in combination with a substitution of lysine for glutamic acid at codon 182 at E2, or deletion of five amino acids at the furin cleavage site in combination with a substitution of lysine for
20 glutamic acid at codon 181 at E2. Additionally, certain mutations placed in the non-coding region at the 5' end of the genome have been found to be attenuating, specifically, a C to T change at nucleotide 7, an A to G change at nucleotide 13, a T
25 to A change at nucleotide 25 and deletion of an A at nucleotide 22. These novel attenuating mutations may be inserted together in a cDNA clone encoding WEE virus resulting in an attenuated WEE which is reflected by 100% survival of mice inoculated by
30 subcutaneous and intracranial routes. Such an attenuated live virus is immunogenic and protective against a lethal virus challenge.

Novel attenuating mutations can be discovered in the VEE IE by introducing mutations which are not
35 repairable by the viral RNA replication process. A

preferable mutation is the deletion of the four amino acids of the furin-like cleavage site between the E3 and E2 proteins. Transfection of the mutant viral genome into cells can result in the suppression of the lethal effect of the deletion mutation due to the error prone process of alphavirus replication. Once efficiently replicating viral progeny is generated they can be detected by plaque assays and analyzed for the presence of pE2 protein which indicates that the virus contains the deletion mutation. Attenuated but yet immunogenic virus with a cleavage deletion mutation and suppressor mutation(s) could be tested for its ability to protect animals from challenge with virulent VEE IE.

Attenuating mutations may be introduced into cDNAs encoding live WEE or VEE IE by any suitable means, such as site-directed mutagenesis (Please see e.g., Maniatis, Fritsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) or Current Protocols in Molecular Biology, Ausubel, F. M et al. (Eds.) John Wiley & Sons, Inc., for general cloning methods.).

In another embodiment of the present invention is provided a chimeric virus containing nonstructural sequences from one alphavirus and structural sequences from other alphaviruses which could be used as a means of attenuating virulent alphaviruses. By "Structural sequences" as used herein is meant sequences encoding proteins which are required for encapsidation (e.g., packaging) of the viral genome, and include the capsid protein, E1 glycoprotein, and E2 glycoprotein. By "nonstructural sequences" is meant nonstructural protein sequences, or sequences which encode viral RNA

polymerase(s) proteins. Viruses from which structural sequences can be used in the chimeric virus using WEE "nonstructural genes" as the backbone clone can include for example, all strains of WEE, EEE, and

5 Sindbis, Aura, Barmah Forest, Bebaru, Bijou Bridge, Cabassou, Chikungunya, Everglades, Fort Morgan, Getah, Highlands J, Kyzylgach, Mayaro, Middelburg, Mucambo, Ndumu, O'nyong-nyong, Pixuna, Ross River, Sagiyama, Semliki Forest, SAAR87, Tonate, Una, Venezuelan Equine

10 Encephalitis, Whataroa, to name a few. Acceptable structural protein genes would include a nucleocapsid protein capable of both packaging the chimeric viral genome and which can interact with the glycoproteins to initiate particle assembly. Chimeric virus is

15 constructed by excision of the structural protein genes of the backbone virus and replacement with the desired structural protein genes from another virus. This can be accomplished in one of several ways. For example, site-directed mutagenesis can be used to

20 excise the structural protein genes and leave a restriction endonuclease digestion site at the point of deletion. The structural protein genes of another alphavirus would then be cloned into that restriction site. Any virus obtained after transfection of cells

25 with RNA transcribed from that clone would by definition be a chimeric virus.

In the case where the first and second viruses are closely related, another method can be used wherein cloned structural cDNA sequences of a second

30 alphavirus can be digested at restriction enzyme sites which both viruses have in common. The cDNA fragments of the second virus can then be cloned into the homologous sites in the first virus structural protein locus such that the resulting structural protein genes

35 of the chimeric are a composite of both. Other

methods for producing a chimeric virus are known to people in the art (Kuhn et al. [1996] *J. Virology* 70:7900-7909).

In another embodiment, the attenuated viruses of the present of invention can be used to prepare replicon expression systems. A replicon expression system consists of three components. The first is a replicon which is equivalent to a full length infectious clone from which all of the viral structural proteins have been deleted. A multiple cloning site can be cloned into the site previously occupied by the structural protein genes. Virtually any heterologous gene may be cloned into this cloning site. Transcription of RNA from the replicon yields an RNA capable of initiating infection of the cell identically to that seen with the full-length infectious virus clone. However, in lieu of the viral structural proteins, the heterologous antigen is expressed. This system does not yield any progeny virus particles because there are no viral structural proteins available to package the RNA into particles.

Particles which appear structurally identical to virus particles can be produced by supplying structural proteins for packaging of the replicon RNA in trans. This is typically done with two helpers. One helper consists of a full length infectious clone from which the nonstructural protein genes and the glycoprotein genes are deleted. The helper retains only the terminal nucleotide sequences, the promoter for subgenomic mRNA transcription and the sequence for the viral nucleocapsid protein. The second helper is identical to the first except that the nucleocapsid gene is deleted and only the glycoprotein genes are retained. The helper RNA's are transcribed *in vitro* and co-transfected with replicon RNA. Because the

replicon RNA retains the sequences for packaging by the nucleocapsid protein, and because the helpers lack these sequences, only the replicon RNA is packaged by the viral structural proteins and released from the cell. The particles can then be inoculated into animals similar to parent virus. The replicon particles will initiate only a single round of replication because the helpers are absent, they produce no progeny virus particles, and express only the viral nonstructural proteins and the product of the heterologous gene cloned in place of the structural proteins. The heterologous gene product is then detected by the host immune system and appropriate immune response is then mounted.

The WEE and VEE IE replicons can be used to express heterologous genes of interest as well as a means for expressing antigens or immunogenic proteins and peptides of interest, *in vitro* or *in vivo*. The immunogenic protein or peptide, or "immunogen" may be any immunogen suitable for inducing an immune response protective against a pathogen from which the immunogen is derived, including but not limited to microbial, bacterial, protozoal, parasitic, and viral pathogens. For example, the immunogen can be the expression product of any heterologous gene of interest, including influenza hemagglutinin, lassa fever nucleocapsid and glycoproteins, portions of bacterial toxin genes, HIV glycoprotein, Ebola glycoprotein, to name a few.

In yet another embodiment, the present invention provides inactivated virus vaccines produced from live attenuated virus preparations, either as virus with attenuating mutations as has been described for WEE and VEE IE or chimeric viruses described above for EEE and VEE IIIA. The inactivation of live virus is well

known in the art and can be performed, for example, by the use of formalin. Inactivated attenuated virus vaccine has a greater safety margin both as a final vaccine in case of incomplete inactivation, and during
5 the manufacturing process allowing production under lower biocontainment levels.

Subjects which may be administered the live attenuated or inactivated attenuated viruses and vaccine formulations disclosed herein include both
10 humans and animals (e.g. horse, donkey, pigs, mice, hamster, monkey, birds).

Vaccine formulations of the present invention comprise an immunogenic amount of a live attenuated virus, or a combination of live attenuated viruses as
15 a multivalent vaccine, as disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the attenuated virus sufficient to evoke an immune response, particularly an immune response to the
20 protein or peptide encoded by the heterologous RNA carried by the virus, in the subject to which the virus is administered. An amount of from about 10^1 to 10^5 plaque forming units of the live virus per dose is suitable, depending upon the age and species of the
25 subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Administration of the live attenuated viruses
30 disclosed herein may be carried out by any suitable means, including both parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), by in ovo injection in birds, and by topical application of the virus (typically carried in
35 the pharmaceutical formulation) to an airway surface.

Topical application of the virus to an airway surface can be carried out by intranasal administration (e.g. by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally). Topical
5 application of the virus to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical formulation (including both solid particles and liquid particles) containing the virus as an aerosol
10 suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of pharmaceutical formulations are well known, and any conventional technique can be employed.

15 In another embodiment, the present invention relates to antibodies specific for the above-described virus. For instance, an antibody can be raised against any of the viral proteins or against a portion thereof. Persons with ordinary skill in the art using
20 standard methodology can raise monoclonal and polyclonal antibodies to a polypeptide of the present invention. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and
25 Practice, Chapter 4, 1986). The antibodies can be used to monitor the presence or activity of alphaviruses and potentially as a therapeutic agent.

In a further embodiment, the present invention relates to a method of detecting the presence of WEE,
30 EEE, VEE IIIA or VEE IE viral infection or antibodies against these viruses, if present, in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a

microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of WEE, EEE, VEE IIIA or VEE IE virus described above, and contacting it with the serum of a person suspected
5 of having a viral infection. The presence of a resulting complex formed between the virus and antibodies specific therefor in the serum can be detected by any of the known methods common in the art, such as colorimetry or microscopy. This method
10 of detection can be used, for example, for the diagnosis of WEE, EEE, VEE IIIA and VEE IE viral infections.

In yet another embodiment, the present invention relates to a method of detecting the presence of WEE,
15 EEE, VEE IIIA or VEE IE viruses in a sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g.
20 nitrocellulose membrane), antibodies specific for WEE and/or VEE IE, and contacting it with serum or tissue sample of a person suspected of having a WEE or VEE IE viral infection. The presence of a resulting complex formed between virus in the serum and antibodies
25 specific therefor can be detected by any of the known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of WEE, EEE, VEE IIIA and VEE IE viral infection.

30 In another embodiment, the present invention relates to a diagnostic kit which contains WEE, EEE, VEE IE, or VEE IIIA virus and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence of antibodies to WEE and
35 VEE IE in serum or a tissue sample. Tissue samples

contemplated can be obtained from birds, monkey, human, or other mammals.

In yet a further embodiment, the present invention relates to DNA or nucleotide sequences for use in detecting the presence or absence of WEE, EEE, VEE IE or VEE IIIA virus using the reverse transcription-polymerase chain reaction (RT-PCR). The DNA sequence of the present invention can be used to design primers which specifically bind to the viral RNA for the purpose of detecting the presence, absence, or quantitating the amount of virus. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides. Reagents and controls necessary for PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of viral sequences, for example by gel fractionation, with or without hybridization, by radiochemistry, and immunochemical techniques.

In yet another embodiment, the present invention relates to a diagnostic kit which contains PCR primers specific for WEE, EEE, VEE IE or VEE IIIA and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence or absence of WEE, EEE, VEE IE or VEE IIIA in a sample using PCR. Samples contemplated can be obtained from birds, human, or other mammals.

In another embodiment, the present invention relates to a method of reducing WEE, EEE, VEE IE, or VEE IIIA viral infection symptoms in a patient by administering to said patient an effective amount of anti WEE, anti EEE, anti VEE IE, or anti VEE IIIA antibodies, or protective serum from an immunized animal. When providing a patient with antibodies, the dosage of administered agent will vary depending upon

such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds
5 which is in the range of from about 1pg/kg to 10 mg/kg body weight of patient, although a lower or higher dosage may be administered.

In another embodiment, the present invention relates to a method for overcoming vaccine
10 interference in alphavirus-immune individuals. Alphavirus interference has been documented in animals and people since the 1960's. This phenomenon occurs when a live-attenuated vaccine is administered to animals or people with existing immunity to
15 heterologous alphaviruses. Pre-existing immunity may be acquired by vaccination or infection. This presents a significant limitation to the usefulness of the current live-attenuated alphavirus vaccines, especially since the cross-reactive immunity does not
20 protect adequately against challenge with virulent heterologous alphaviruses. Formalin-inactivated vaccines are not an acceptable alternative as they have significant limitations with regard to the quality and duration of protective immunity and
25 require multiple inoculations and periodic boosters. The attenuated WEE, EEE, VEE IIIA and VEE IE virus vaccines of the present invention contain mutations in the viral glycoprotein sequences that may alter the sequence, conformation, and/or accessibility of cross-
30 reactive epitopes. Alterations in epitopes that prevent binding by cross-reactive antibodies may also bypass interference in alphavirus-immune individuals. Eliminating the problem of interference would permit the WEE and VEE IE attenuated virus vaccines to be
35 used in alphavirus-immune animals or people to induce

protective immunity to western equine encephalitis virus and/or to Venezuelan equine encephalitis virus variant IE. Long-lasting protective immunity to both parenteral and aerosol challenge would be expected
5 after vaccination with the live-attenuated vaccines of the present invention, and provide an additional advantage over the use of inactivated vaccines which induce short-lived responses that do not protect against mucosal challenge.

10 Having now described the invention, the following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary
15 skill in the art.

Attenuated WEE

The following materials and methods were used in
20 the examples that follow.

Viruses and cells. Western equine encephalitis virus, strain CBA/87 (Bianchi et al. 1988) and eastern equine encephalitis virus, strain Fla91-4679 (Mitchell et al. [1992] *Science* 257:526-7) were grown in BHK
25 cells in EMEM containing 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin. Primary chicken embryo fibroblasts were grown in EMEM containing 5% fetal bovine serum. Baby hamster kidney cells and Vero cells were grown in EMEM containing 10%
30 fetal bovine serum.

cDNA cloning. Genomic RNA was prepared from purified virus by phenol:chloroform extraction and ethanol precipitation. Initially, cDNA was prepared by the method of Gubler and Hoffman ([1983] *Proc.*

Natl. Acad. Sci. U.S.A. 85:5997-6001). Subsequently, DNA representing the entire genome was prepared by polymerase chain reaction using a series of primer pairs (Table 1, Figure 1) based upon the partial genome sequences previously deposited in GenBank. Each PCR product was cloned into pCRII (Invitrogen). The 5' terminal sequence of CBA/87 virus was determined by 5'-RACE basically as described by Frohman et al. ([1988] Proc. Natl. Acad. Sci. U.S.A. 85:8998-9002). The oligonucleotide, CBA/87 5', consisted of an SstII site, the promoter for bacteriophage T7 RNA polymerase followed by one G and 14 nucleotides of the 5' terminus of CBA/87 terminal sequence was paired with ns1962 and used to amplify the terminal 1.9 kb of the WEE genome. Clone pWE2000 representing the entire genome was assembled in pBluescript KS+ through the use of convenient restriction endonuclease sites.

20 Table 1. Oligonucleotide Primers for Preparation of WEE PCR Products

	CBA/87 -T7-Sst2	GTCACCGCGGTAATACGACTCACTATAGATAGGGCATGGTATAGAG (SEQ ID NO:4)
25	NS1962	TCACCTTATTCTGGAACACATCAG (SEQ ID NO:5)
	WEE-7	TCGGAGGAAGGCTGATGAAAC (SEQ ID NO:6)
	WEE-10	TCGGATCCGATGAGAAAATATACGCTCCC (SEQ ID NO:7)
	WEE-17	GACTGGATCCGCAAACCAGTCCTGTTCTCAGG (SEQ ID NO:8)
30	WEE-18	GCATGGATCCAGCATGATCGGAAATGTCTTGTC (SEQ ID NO: 9)
	WEE-5	TCGGATCCACCGCCAAAATGTTTCCATAC (SEQ ID NO:10)
	WEE-3	TCGGGATCCCCGGAACATTTGGC (SEQ ID NO:11)
	WEE-2	CTGCTTTTCATGCTGCATGCC (SEQ ID NO:12)
35	WEE-Not	CGATGCGGCCGCTTTTTTTTTTTTTTTTTTTGAAATTTTAAAAAC (SEQ ID NO:13)
	WEE-CL2	CAGCGTGAAGTCATCGGTAATGCTGCGTGATGGACATTTCAAG (SEQ ID NO:14)
40	WEE-CL1	CAGCGTGAAGTCATCGGTAATGCTTGATGGACATTTCAAG (SEQ ID NO:15)

For ease of subsequent site-directed mutagenesis of the structural protein genes, two cassettes representing the 5' terminal 7.6 kb, plasmid pWE5'-18, and 3' 4.2 kb, plasmid pWE3'-17, of the genome were prepared. Full length clones were assembled by digestion of the pWE5'-18 with *BlnI* and *NotI* and insertion of a 4.1 kb *BlnI-NotI* fragment prepared from the plasmid pWE3'-17 or its mutagenized derivatives.

A cassette containing the structural genes of eastern equine encephalitis virus strain Fla91-4679 was prepared by RT-PCR. The cassette was digested with *BlnI* and *NotI* and the 4.0 kb fragment was ligated to pWE5'-18 which had been similarly digested. The resulting plasmid was designated pMWE-7.

Mutagenesis of the furin cleavage site. Two oligonucleotides, WEE-CL2 and WEE-CL which bracket the presumed furin cleavage site, RRPKR, between the E3 and E2 glycoproteins were used to delete the 5 and 4 codons, respectively. Plasmid pWE3'-17 was used as template for mutagenesis. WEECL2 and WEECL were paired with primer WEE-5 to generate PCR products of 1.4 kb. The PCR products were purified and paired with WEE-3 for 10 cycles of PCR utilizing WE3'-17 as template. Additional WEE-5 primer was added to 500 nM and PCR was carried out for an additional 20 cycles. The 2.3 kb products were purified, digested with *BstEII* and *NcoI* and ligated into plasmid pWE3'-17 which had been digested with *BstEII* and *NcoI*. Clones containing the mutation were identified by loss of the *NgoM1* site in the sequences encoding the furin cleavage site. The sequences of the mutations were confirmed by sequencing and the mutagenized pWE3'-17 cassettes were ligated to pWE5'-18 as described above to yield full length clones pWE2200 and pWE2100, respectively.

Identification of secondary mutations in virus derived from pWE2100 and pWE2200. Virus released from cells electroporated with RNA transcribed from pWE2200 were plaque-purified and grown into stock
5 preparations. Supernatant from cells transfected with RNA transcribed from pWE2100 was diluted 10-fold and inoculated onto BHK cells. The supernatant was collected 48 hours later and the RNA was extracted directly from the culture fluid. For each mutant
10 virus and the parent CBA/87 virus, viral RNA was extracted with Trizol LS (Life Technologies, Inc., Gaithersburg, MD) and the glycoprotein genes of each were amplified by reverse transcription-polymerase chain reaction amplification. The PCR products were
15 purified (PCR Prep, Promega Inc., Madison, WI) and sequenced on an ABI 373 sequencer using fluorescent-tagged terminators.

Transcription and transfection. Purified plasmid was digested with NotI, phenol extracted and ethanol
20 precipitated. Typically 0.5-1 ug of linearized DNA was transcribed in vitro by T7 RNA polymerase (Ribomax, Promega, Madison, WI) in the presence of 3 mM m7GpppGp (Pharmacia, Piscataway, NJ). Electroporation of BHK or CEF cells with 0.4 ug of RNA
25 was done as described (Liljestrom et al. [1991] *Bio/Technology* 9:1356-1361). The cells were then seeded into T-75 flasks with 20 ml of medium and observed for cytopathology at 24 and 36 hours after electroporation. Virus was harvested when the cells
30 displayed significant cytopathology and approximately 50% were detached from the plastic. Virus titers were determined by plaque assay on Vero and BHK cells.

EXAMPLE 1**Preparation of an infectious clone of WEE strain CBA/87.**

One goal of this study was to prepare a full
5 length cDNA clone of fully virulent WEE virus such
that mutations leading to an attenuated phenotype
could be identified. WEE, strain CBA/87, isolated
from the brain of an infected horse in Argentina in
1987 (Bianchi et al. [1988] *Am. J. Trop. Med. Hyg.*
10 **49:322-328**), was chosen as the parent virus as it
consistently kills 100% of 5 week old C57BL6 mice when
inoculated subcutaneously, allowing development of a
convenient animal model to assess the relative effects
on virulence of the attenuating mutations. Portions
15 of the sequence of several strains of WEE virus had
been determined previously and were used as a basis
for primers to prepare amplification products
representing the entire genome of the CBA/87 which
were cloned in pCRII. Full length clones were
20 assembled in pBluescript KS+ using convenient
restriction sites as shown in Figure 1. The first
infectious clone pWE1000, contained the 5' terminal 20
nucleotides from eastern equine encephalitis virus.
This clone produced viable virus which was highly
25 attenuated exhibiting an subcutaneous LD₅₀ in mice of
approximately 1.2 X 10⁶ PFU compared to the CBA/87
parent virus where LD₅₀ was approximately 22.

A second clone with an authentic WEE 5'-terminus,
pWE2000, was used for all subsequent experiments.
30 Transfection of CEF or BHK cells with RNA transcribed
from pWE2000 resulted in complete destruction of the
monolayers within 36 hours and titers >10⁸ PFU/ml were
obtained by infection of either cell type with the
resulting virus. Subcutaneous inoculation of 5 week

old C57/Bl6 mice with WE2000 results in death within 9 days and the LD₅₀ of 75 PFU is only slightly higher than the LD₅₀ of 22 PFU of CBA/87 parent virus, Table 2. It should be noted that animals surviving the lower doses of virus challenge gave no serological evidence of infection. This level of virulence for WE2000 virus was considered sufficient to allow for further characterization of the mutations necessary for attenuation of the virus.

10

Table 2. C57 Black/6 Mice Inoculated Subcutaneously with CBA/87 Parental virus and WE2000 Recombinant Virus

<u>Virus Strain</u> Dose	Mortality	Mean Day to Death (Days)	Prechallenge ELISA	Challenge (S/T)
<u>CBA/87</u>				
3X10 ⁵	10/10	8.4	-	-
3X10 ⁴	10/10	8.9	-	-
3X10 ³	10/10	9.2	-	-
3X10 ²	7/10	9.9	<100	0/3
3X10 ¹	3/10	10	<100	0/7
3	0/10	--	<100	0/10
<u>WE2000</u>				
1X10 ⁷	10/10	8.7	-	-
1X10 ⁶	10/10	9.0	-	-
1X10 ⁵	10/10	9.2	-	-
1X10 ⁴	10/10	9.3	-	-
1X10 ³	9/10	10.0	<100	0/1
1X10 ²	3/10	10.0	<100	0/7
1X10 ¹	0/10	-	<100	0/10
1	0/10	-	<100	0/10

15

EXAMPLE 2

Preparation of cleavage mutants of CBA/87.

Davis et al. ([1995] *Virology* 212:102-110) have demonstrated that deletion of the furin cleavage site between E3 and E2 glycoproteins of VEE virus is a lethal mutation. However, prolonged incubation of cells which had been transfected with RNA derived from

20

full length clones with the deletion, resulted in the eventual appearance of virus which was replication-competent and attenuated in mice (Davis *et al.*, 1995, *supra*). Based upon a comparison of the predicted structural protein sequences of WEE and VEE, the probable cleavage site of CBA/87 virus is RRPKR. The presence of the extra arginine when compared to the consensus (RX(R/K)R) alphavirus cleavage site indicated that the cleavage site of WEE virus might be more complex than that observed with VEE virus. We therefore, prepared two different deletion mutations in the E3-E2 cleavage site of the pWE2000 clone, pWE2100 which lacks five amino acids, RRPKR, and pWE2200 which lacks four amino acids, RPKR.

Development of CPE in cells after electroporation of RNA transcribed from pWE2100 and pWE2200 was delayed for 48 to 72 hours compared with pWE2000. In two instances, pWE2100 did not induce significant CPE in the transfected cells despite the fact that approximately 10^6 PFU/ml were released into the medium of transfected cells. When assayed by plaque formation on Vero cells, both supernatants yielded extremely small plaques after 72 hours which never increased beyond 2 mm in diameter. In contrast, pWE2000 virus yields large plaques after 48 hours which enlarge to approximately 1 cm after 5 days under a 0.5% agarose overlay. The small plaque phenotype of the mutant viruses is stable after 3 passages in Vero or BHK cells, which is the limit to the passage of the virus used in these experiments.

Analysis of the structural proteins of the WE2100 and WE2200 viruses by SDS-PAGE (Figure 2) shows that in each instance, the deletions at the cleavage site result in a virus which lacks E2 protein and contains a larger protein, presumably pE2, indicating that

deletion of the presumed cleavage site eliminated cleavage at this site.

The lack of rapid cytopathology after transfection of BHK cells with transcripts of pWE2200 and pWE2100 suggested that the mutants were non-viable and that the infectious virus subsequently detected by plaque assay was due to secondary mutations arising during the replication of the RNA as reported previously for deletion mutants of VEE (Davis et al., 1995, *supra*).

Three plaque isolates from WE2200 virus were chosen for further characterization. All isolates grew to high titer and exhibited a small plaque phenotype. The isolates were sequenced over the entire glycoprotein reading frame. As shown in Table 3, isolates 2215 and 2220 have a mutation of Glu to Lys at position 181 of the E2 glycoprotein. Strain WE2219 carries a single Glu to Lys at position 182 of the E2 protein. Strain WE2215 also has a conservative Val to Ala change at position 211 of the E2 glycoprotein. WE2220 has a Glu to Gly change at position 2 of the E1 glycoprotein and a Phe to Ser change at position 382 of the E1 glycoprotein.

Table 3. Genotypes of Recombinant WEE Virus Strains

<u>Virus</u> ¹	<u>Cleavage Site</u>	<u>E2</u> ²	<u>E1</u> ²
CBA/87	RRPKR	P(102),E(181),E(182)	E(2),F(257),P(382)
vWE2100	-----	K(182)	
pWE2102	-----	K(182)	
vWE2215	R-----	K(181), A(211)	
vWE2219	R-----	K(182)	
vWE2220	R-----	K(181)	G(2), S(382)

1. p indicates viruses prepared by mutagenesis of infectious clones.
2. Amino acid at position indicated in parenthesis.

5 In order to determine a consensus of the
mutations appearing in virus produced from the pWE2100
RNA, the sequences of the glycoprotein genes were
determined directly from cDNA prepared by RT-PCR of
the genomic RNA extracted from virus released from BHK
10 cells infected with virus released from the
transfected cells. Sequence analysis of glycoprotein
genes of WE2100 virus from the transfection
supernatant revealed only two mutations. As seen
previously in WE2219, WE2100 also had a Glu to Lys
15 change at position 182 of the E2 glycoprotein (Table
3).

 In order to determine which of the mutations
identified in virus released from cells transfected
with RNA transcripts from pWE2200 and pWE2100 served
20 as the suppressor of the lethal effect of the cleavage
deletion mutation, the mutations were individually
placed into the pWE2200 and pWE2100 clones by site-
directed mutagenesis. As shown in Table 4, three
subclones of WE2200 were produced, and based upon the
25 ability to induce CPE in BHK cells, it was
demonstrated that the Glu to Lys change at position
181 of the E2 glycoprotein was necessary and
sufficient to restore the ability of the WE2200 clone
to encode replication competent virus. Similarly,
30 placement of the Glu to Lys change at position 182 of
the E2 glycoprotein was also sufficient to restore the
ability of the WE2100 clone to encode replication
competent virus. When the Glu to Lys changes at E2
position 181 or 182 were inserted into the parental
35 infectious clone pWE2000, the resulting virus

exhibited a small plaque phenotype on Vero cells as noted for each of the cleavage deletion mutants.

5 Table 4. Effect of site-directed mutagenesis on restoration of cytopathogenicity of pWE2200 and pWE2100

Strain	Cleavage site	E2	E1	Viability
10 pWE2000	RRPKR			Yes
pWE2200	R----			No
pWE2221	R----		G(2)	No
15 pWE2222	R----	K(181)	G(2)	Yes
pWE2223	R----	K(181)		Yes
pWE2100	-----			No
pWE2102	-----	K(182)		Yes

20

Although the cleavage deletion mutations in pWE2200 and pWE2100 differed by a single amino acid, the results indicate that the mutations at E2 residues 181 and 182 are both capable of restoring viability of
25 the virus and appear to be equivalent.

EXAMPLE 3

Attenuation of WE2000 virus.

30 C57/BL6 mice are uniformly susceptible to lethal challenge by western equine encephalitis virus until approximately 9 weeks of age. Subcutaneous inoculation of five- or eight-week-old C57/BL6 female mice with CBA/87 or WE2000 viruses routinely results in lethal encephalitis after 8-9 days (Table 2). As
35 noted previously, the WE2000 is slightly less virulent than the CBA/87 parent. The virulence of the progeny virus derived from pWE2100 and pWE2200 infectious clones were determined by subcutaneous inoculation of C57BL6 mice. In each instance, the viruses were

significantly attenuated compared to virus produced from parent WE2000 clone. However, infection of mice with increasing doses of WE2215, WE2219 or WE2220, resulted in sporadic deaths with slightly extended periods prior to death compared to the parental virus (Table 5). These results indicated that deletion of only four amino acids from the cleavage site was inadequately attenuating, unlike the results obtained with VEE virus by Davis *et al.* (1995) and the viruses derived from WE2200 were not characterized further.

Table 5. C57BL6 Mice Inoculated Subcutaneously with WE2200 Cleavage Deletion Mutants

<u>Virus Strain</u> Dose	Mortality	Mean day to Death	Prechallenge ELISA	Neut	Challenge length	Post Challenge ELISA	Neut
<u>CBA/87</u>							
10 ³	10/10	9.1					
10 ⁵	10/10	7.7					
<u>WE2000</u>							
10 ³	10/10	9.2					
10 ⁵	10/10	9.1					
<u>WE2215</u>							
10 ³	1/10	12	504 (3/9) 100 (6/9)	<20 (9/9)	8/9	8300	761
10 ⁵	1/10	14	253 (7/9) 100 (2/2)	<20 (9/9)	9/9	12800	403
<u>WE2219</u>							
10 ³	0/10	-	283 (2/8) 100 (6/8)	<20 (10/10)	6/10	14368	2281
10 ⁵	2/10	12	200 (4/8) 100 (4/8)	<20 (8/8)	8/8	11738	1522
<u>WE2220</u>							
10 ³	1/10	11	606 (5/9) 100 (4/9)	20 (3/9) <20 (6/9)	5/9	14703	1280
10 ⁵	2/10	14	800 (7/8) 100 (1/8)	20 (2/8) <20 (6/8)	8/8	9051	761

When C57BL6 mice were inoculated subcutaneously with the uncloned WE2100 progeny virus, there were no deaths at any dilution, even at doses of 10^7 PFU per mouse (Table 6). However, some of those mice inoculated subcutaneously with 10^5 PFU or less remained susceptible to a lethal challenge with the virulent CBA/87 virus.

10 Table 6. C57 Black/6 Mice Inoculated Subcutaneously with WEE Virus Strain WE2100

Virus strain Dose	Mortality ¹ (%)	Mean Day to Death	Challenge ² (%)
<u>WE2100</u>			
10^3	0/10 (0)	-	4/10 (40)
10^4	0/10 (0)	-	7/10 (70)
10^5	0/10 (0)	-	8/10 (80)
10^6	0/10 (0)	-	10/10 (100)
10^7	0/10 (0)	-	10/10 (100)

- 15 1. Expressed as animals dying/animals tested
 2. Expressed as animals surviving/animals tested

WE2102 virus was demonstrated to be highly attenuated and killed only two of twenty mice when inoculated subcutaneously with the highest dosage of virus (10^7 PFU). All mice were challenged 3 weeks later with 10^5 PFU of CBA/87 virus. Mice previously immunized with 10^5 PFU or more of WE2102 survived with no noticeable symptoms. Thus, an effective immunizing dose of WE2102 is at least 100 fold less than that required to kill C57BL6 mice. These results further indicate that the Glu to Lys change at position 182 of the E2 glycoprotein is responsible for restoring viability to viruses containing a deletion of the furin cleavage site in the WEE glycoproteins and that WE2102 virus is an effective attenuated vaccine virus.

Table 7. Subcutaneous Inoculation of C57BL/6 Mice with recombinant WEE virus strain 2102 confers protection against a lethal challenge

5

<u>Virus Strain</u>	<u>Mortality¹</u> (%)	<u>Mean Day to Death</u>	<u>Challenge²</u> (%)
<u>Dose</u>			
<u>WE2100</u>			
10 ³	0/10 (0)		3/10 (30)
10 ⁵	1/10 (10)	16	9/9 (100)
10 ⁷	0/10 (0)		10/10 (100)
<u>WE2102</u>			
10 ³	0/20 (0)	-	9/20 (45)
10 ⁵	0/20 (0)	-	18/20 (90)
10 ⁷	2/20 (0)	14	18/18 (100)
<u>WE2000</u>			
10 ⁵	10/10 (100)	9.2	

1. Expressed as animals dying/animals tested
2. Expressed as animals surviving/animals tested

10

Attenuated VEE IE

EXAMPLE 4

Sequence Analysis. Genomic RNA from VEE IE Strain 68U201 was isolated and reverse transcription of the genomic RNA followed by polymerase chain reaction (RT-PCR) was used to generate cDNA of the virus. Initial sequencing of the strain 68U201 genome employed oligonucleotide primers based on existing VEE IE sequence and VEE IA/B sequence. After a partial sequence of strain 68U201 was determined, oligonucleotides specific to the strain 68U201 sequence were used to obtain a complete sequence of the strain 68U201 viral genome. The exact 5' end of the genome was determined by PCR/RACE technique (Frohman et al. [1988] *Proc. Natl. Acad. Sci. U.S.A.* 85:8998-9002). The entire strain 68U201 viral genomes consists of 11,464

nucleotides, excluding the poly-A sequence (Oberste et al. [1996] *Virology* 219: 314-320).

EXAMPLE 5

5 Construction of Full-length, Live Clones

Using oligonucleotides specific to the VEE IE strain 68U201 sequence, RT-PCR of strain 68U201 viral RNA was carried out to generate numerous cDNA fragments that were subsequently cloned. These clones
 10 were used to assemble a full-length cDNA of strain 68U201 in a plasmid situated such that the cDNA could be precisely transcribed in an *in vitro* transcription reaction employing T7 polymerase. The first
 15 nucleotide downstream of the T7 promoter is a G followed by a cDNA encoding the entire strain 68U201 genome, including poly-A sequence. For the purposes of run off *in vitro* transcription, a unique
 endonuclease restriction site (*NotI*) follows the poly-A sequence (Figure 4). DNA sequences encoding the T7
 20 promoter and the strain 68U201 genome were cloned into a suitable plasmid for propagation and selection in *E. coli*. Oligonucleotides relevant to construction of full-length infectious clones are shown in Table 8.

25 Table 8

	0077	CTAAGAGGGGCCCTATATC (SEQ ID NO:16)
	0111	GCGGAATTCTAATACGACTCACTATAGATGGGCGGCGCATGAGAG (SEQ ID NO:17)
30	0113	TGACCGCGGGACCTCTGTCCAC (SEQ ID NO:18)
	0126	AAGTGCATCGATTTCAGCG (SEQ ID NO:19)
	0136	CTGAAATGTCCAGGATCCACGGAGGAGCTG (SEQ ID NO:20)
	0137	CAGCTCCTCCGTGGATCCTGGACATTCAG (SEQ ID NO:21)
	0140	GACTGCGGCCGCTTTTTTTTTTTGAAATATTAAAAACAAAATCC (SEQ ID NO:22)
35	0220	CGAGAATCGATGCACTTCAGCC (SEQ ID NO:23)

Descriptions of oligonucleotides are as follows:
0077 introduces an *ApaI* endonuclease restriction site
along with a serine to proline mutation within the
coding region of nsP4. 0111 encodes an *EcoRI*
5 endonuclease restriction site followed by the T7
promoter, a single G, and the first 18 nucleotides of
the VEE IE genome. 0126 introduces a *ClaI*
endonuclease restriction site within the coding region
of nsP3 and was used in combination with 0220. 0113
10 was used to remove an *ApaI* endonuclease restriction
site within the structural genes. 0140 encodes a
unique *NotI* endonuclease restriction site followed by
a 12 T's, and the reverse complement of the last 21
nucleotides of the VEE IE. A *NotI* site at the end of
15 the cDNA encoding the VEE IE genome allowed for run-
off transcription after digestion of the plasmid with
NotI. 0220 introduces a *ClaI* endonuclease restriction
site within the coding region of nsP3 and was used in
combination with 0126. Minor alterations of the
20 nucleotide sequence using 0077, 0113, 0126, and 0220
facilitated assembly of the full-length clones and
allowed for rapid diagnostic analysis of virus
generated from these clones by RT/PCR methods.

The full-length clone obtained, pIE1006, was
25 transcribed *in vitro* using T7 polymerase of *NotI*-
linearized plasmid and the RNA transfected into BHK-21
cells (Figure 4). The phenotype of the resulting
virus was markedly different from the parent virus
(strain 68U201) from which the cDNA was derived. The
30 virus derived from pIE1006 *in vitro* transcribed RNA,
VIE1006, gave rise to small plaques upon infection of
target monolayers (Table 9). In attempts to recover
the phenotypic characteristics of strain 68U201,
regions of the pIE1006 clone were replaced with cDNA
35 generated by RT-PCR from strain 68U201 RNA. Three

subsequent full-length, live clones were constructed by replacing pIE1006 sequences with 928, 4492, and 8130 nucleotides generated by RT-PCR of strain 68U201 RNA. These clones were designated pIE1007, pIE1008
5 and pIE1009; and viruses derived from these clones were designated VIE1007, VIE1008 and VIE1009, respectively. These clones are shown in Figure 4. The relevant cloning sites and genetic markers are indicated above each clone. Numbers indicate the
10 corresponding nucleotides in the strain 68U201 genome. The T7 promoter is shown. Drawings are not to scale. Shaded areas represent regions of pIE1006 that were replaced or mutated to generate new clones. The characteristics of these viruses are described below.

15

EXAMPLE 6

Assessment of Viruses Derived from Various Clones. The viruses VIE1006, VIE1007, VIE1008 and VIE1009 were derived from plasmids pIE1006, pIE1007,
20 pIE1008, and pIE1009, respectively. Analysis of virus derived from these clones by plaque assay is shown in Table 9. Plaque size was determined by infection of Vero cell monolayers followed by agarose overlay.

Analysis of virus derived from these clones
25 (Table 9) by plaque assay indicated that VIE1007 gave the same plaque morphology as VIE1006 and therefore was not studied further. The VIE1008 produced larger plaques in comparison to VIE1006, and the VIE1009 virus gave rise to the largest sized plaques of the
30 four viruses tested. Plaques generated by VIE1009 were similar to those produced by the parental virus, strain 68U201.

35

Table 9: In vitro analysis of virus derived from molecular clones of VEE IE strain 68U201

	Virus Strain¹	Plaque Size
5	VIE1006	small
	VIE1007	small
	VIE1008	medium
10	VIE1009	large
	VEE IE 68U201	large

EXAMPLE 7

15 In Vivo studies of VEE IE Vaccine Candidates. The distinct plaque morphologies of VIE1006, VIE1008, and VIE1009 suggested that these viruses may behave differently in vivo. To assess the relative virulence of the cloned derivatives of strain 68U201, mice were initially inoculated with subcutaneously VIE1006, VIE1008, and VIE1009 viruses (Table 10). Mice infected with VIE1006 and VIE1008 were not adversely affected by these viruses as assayed by the number of mice surviving the infection. VIE1009 proved to be as virulent as strain 68U201, causing death in all of the animals infected (Table 10). Immunogenicity of the different viruses inferred by the demonstration of a protective immune response and was determined by back challenge of surviving animals in the virulence assay with approximately 10^4 pfu of the virulent, parental virus, strain 68U201. Back challenge was performed four weeks after the initial inoculation.

Table 10: In vivo analysis of virus derived from molecular clones of VEE IE strain 68U201

	Virus Strain¹	Mortality²	Challenge³
5	VIE1006	0/10	10/10
	VIE1008	0/10	10/10
	VIE1009	10/10	nd
10	VEE IE 68U201	nd	1/10

¹ Initial inoculation with approximately 10^4 PFU of each virus with the exception of VEE IE 68U201 which were left untreated until challenge phase of experiment.

² Expressed as animals dying/animals tested.

³ Expressed as animals surviving/animals tested. All animals were challenged with approximately 10^4 PFU of VEE IE strain 68U201 four weeks after the initial inoculation.

EXAMPLE 8

Construction of a Full-length, Molecularly Attenuated VEE IE Clone. With the availability of a full-length virulent clone, specific attenuating mutations were introduced into the structural genes of the virus by site-directed mutagenesis. A deletion mutation was used instead of a point mutation because of the inability of viral RNA replication to repair such mutations. The glycoproteins of VEE IE are produced as a poly-protein precursor, PE2. The junction between the E3 and E2 proteins is cleaved by a furin-like cellular protease. The amino acid sequence of the presumed furin-like protease cleavage site of strain 68U201 is RGKR. The nucleotides encoding these four amino acids of the furin-like cleavage site between the E3 and E2

proteins were deleted from the pIE1009 clone and the resulting "cleavage deletion" clone was designated pIE1100. The oligonucleotides used to generate the cleavage deletion mutation are shown in Table 8.

5 Oligonucleotide 0136 encodes a cleavage deletion mutation that eliminates the four amino acid furin-like cleavage site found in PE2 and was used in combination with oligonucleotide 0137. 0137 encodes the reverse complement of 0136.

10 Transfection of RNA transcribed from pIE1100 into tissue culture cells required extended incubation periods before viral cytopathic effect became apparent in the cultured cells. This extended incubation period is indicative of transcripts from full-length
15 clones possessing mutations that partially inhibit viral replication. However, such mutations can be suppressed by second-site mutations which arise randomly via the error prone process of alphavirus replication, resulting in variants with enhanced
20 replication ability. In fact, the culture media from cells transfected with RNA transcribed from pIE1100 contained low titers of infectious virus, VIE1100, which could be amplified to high titer (4×10^7 PFU/ml) upon subsequent passage. Biochemical analysis showed
25 that this virus had an uncleaved PE2 protein indicating that the cleavage deletion mutation totally prevented proteolytic processing of the surface E2 glycoprotein precursor. The efficacy of VIE1100 virus to serve as a vaccine was evaluated in mice as
30 described below.

One specific suppressor mutation is thought to reside at nucleotide 10,181 of the VEE IE genome, a C to U nucleotide substitution resulting in an amino acid change from Serine to Leucine in the E1 protein
35 position #57. Ability of this mutation to suppress the

lethal nature of the furin cleavage site deletion mutation was assessed. The cDNA of pIE1100 was mutated by changing nucleotide 10,181 from a C to T. This introduced a serine to leucine change at amino acid number 57 of E1, a mutation found within the stock of VIE1100. The resulting clone was designated pVIE1150. Transfection of RNA from pVIE1150 into BHK-21 cells lead to the production of approximately 10^6 PFU/ml of virus in the supernatant of the transfected cells at 48 hours post-transfection.

EXAMPLE 9

Vaccine Studies with a Full-length, Molecularly Attenuated Virus. Balb/C mice were inoculated with VIE1100 virus at various doses (10^4 , 10^6 , and 10^7 PFU per mouse), and was found to be completely attenuated at a dose 100,000 times higher than that required to cause lethal disease by the parent virus, strain 68U201 (Table 10). Subsequent challenge of these animals with virulent strain 68U201 demonstrated that immunization with VIE1100 virus provided complete protection from lethal virus challenge (Table 11).

Table 11: Vaccination study with VIE1100

Virus Strain Dose	Mortality ¹	Challenge ²
VIE1100		
10^4	10/10	10/10
10^6	10/10	10/10
10^7	10/10	10/10
Mock Vaccinated	0/10	0/10

1. Expressed as animals dying/animals tested.
2. Expressed as animals surviving/animals tested after inoculation with 10^4 PFU of VEE IE strain 68U201

EXAMPLE 10

Properties of a WEE-EEE chimeric virus. Based upon similarities of amino acid sequences of the carboxy terminal portion of NSP-4 and the amino terminal portion of the capsid proteins of WEE, EEE and Sindbis virus, it has been suggested that WEE virus arose by recombination between EEE and a Sindbis virus ancestor (Hahn et al. [1988] *Proc. Natl. Acad. Sci. U.S.A.* **85**: 5997-6001). The capsid genes of both WEE and EEE contain a highly conserved sequence with a unique Bln I site 76 nucleotides downstream of the initiation codon. We utilized this site to insert the structural protein sequences of EEE into pWE5-18 in order to construct a full length clone encoding a chimeric virus. Plasmid pMWE-7 is a full length clone consisting of the 5' non-coding sequence, nonstructural genes, 26S promoter and the first 25 codons for the capsid protein of WEE CBA/87 fused to the structural protein genes and 3' non-coding sequence of EEE strain Fla91-4679. Transfection of BHK cells with RNA transcribed from pMWE-7 resulted in complete destruction of the monolayer and release of high yields of virus. SDS-polyacrylamide gel electrophoresis of the purified virus demonstrated that the virus is composed of polypeptides which comigrate with those of EEE and not of WEE, indicating that the virus is a chimera (Figure 3).

Injection of mice with the MWE chimeric virus killed mice only sporadically, suggesting that fusion of the sequences of the two viruses resulted in significant attenuation compared to the parent WE2000 virus (Table 12). Mice immunized with MWE-7 developed significant neutralizing antibody titers and resisted a lethal EEE Fla91-1467 challenge when immunized with greater than 10^5 plaque forming units of

MWE-7 virus. The neutralizing antibody response after challenge was not significantly elevated indicating that the immunization with the chimeric virus effectively prevented infection by a lethal EEE challenge. Therefore, a chimeric virus derived by combining the structural protein genes of EEE with the non-structural proteins genes of WEE may serve as a safe, effective approach to development of a vaccine for EEE virus.

10

Table 12. Immunization of C57BL6 Mice with an WEE/EEE Chimeric Virus Confers Protection Against a Lethal EEE Challenge

<u>Virus Strain</u>	<u>Mortality¹</u>	<u>Mean Day to Death</u>	<u>Challenge²</u>
<u>Dose</u>			
<u>WE2000</u>			
10 ⁵	10/10 (100)	8.5	
<u>MWE7</u>			
10 ³	0/10 (0)		6/10 (60)
10 ⁵	1/10 (10)	8.0	9/9 (100)
10 ⁷	0/10 (0) ³		9/9 (100)
<u>Saline Control</u>	0/10 (0)		0/10 (0)

- 15
1. Expressed as animals dying/animals tested
 2. Expressed as animals surviving/animals tested
 3. One animal died during pre-challenge bleed

20

Example 12
VEE IIIA Chimera

Construction of IAB-III A cDNA Chimeric Clone (pV3A-1000)

25 BeAn8 (wild type Mucambo or VEE IIIA) cDNA was amplified with primers 128 and 140 (Table 13) to generate a PCR fragment of the entire 26S region of BeAn8. Primer 128 incorporated an ApaI site just upstream of the BeAn8 26S promoter to facilitate cloning into pV3000. Primer 140 introduced a NotI

30

site just downstream of the poly(A) tract which would also facilitate cloning into pV3000 as well as enable run-off transcription. This 128/140 PCR fragment was cloned into pBluescript SK+ vector using the ApaI and NotI sites and was termed pMUC-1000.

To replace the BeAn8 3' nontranslated region (3'NTR) in pMUC-1000 with the corresponding region in pV3000, an EcoRI site was introduced immediately downstream of the E1 stop codon in both pMUC-1000 and pV3000 using the primer pairs 013/227 and 225/226, respectively. The 013 primer is a universal primer found in the pBluescript SK+ vector and the 227 primer was designed to introduce an EcoRI site in pMUC-1000. The 225 primer introduced an EcoRI site in pV3000 and the 226 primer is located in the vector, TotoX. The 013/227 and 225/226 PCR fragments were separately cloned into the pBluescript SK+ vector producing pMUC-1200 and pV3nt-1000, respectively. The 3' NTR encoded in pV3nt-1000 was shuttled into pMUC-1200 using the EcoRI site and the NotI site to produce pMUC-1300.

The full-length chimeric cDNA clone, pV3A-1000, was constructed by shuttling the structural genes encoded in pMUC-1300 into the pV3000 nonstructural domain using the ApaI site and NotI site.

Table 13. Primers for construction of the pV3A-1000

013 -- AACAGCTATGACCATG (SEQ ID NO:24)
 128 -- CTGAGAGGGGCCCCAGTAAC (SEQ ID NO:25)
 140 -- GACTGCGGCCGCTTTTTTTTTTTTGAATATATAAAA (SEQ ID NO: 26)
 225 -- CCAGAAACATAATTGAATTCAGCAGCAATTG (SEQ ID NO:27)
 226 -- CTTTATCCGCCTCCATCC (SEQ ID NO:28)
 227 -- CCAATCGCTGCTGAATTCTAATTATGTTTCTG (SEQ ID NO:29)

Five week old C57BL6 mice were used in a challenge study (Table 14). All mice immunized with

10⁷ PFU of pV3A-1000 produced neutralizing antibodies. Mice were challenged with 10⁷ PFU of 900807 (a Mucambo virus from Trinidad). The geometric mean neutralizing antibody titer in mice inoculated with 10⁷, 10⁵, and 10³ PFU of pV3A-1000 was 9800, 8800, and 7000 respectively.

Table 14. Results from challenge studies

	Fraction of mice sick/inoc	Fraction of mice dead/inoc	Average days to onset of illness	Average days to death
10				
15	Immunized with pV3A-1000 0/20	0/20	N/A	N/A
20	Unvaccinated 19/20	12/20	6.2	10.5

25

30

35

40

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Michael D. Parker
Jonathan F. Smith
Bruce Crise
Mark Steve Oberste
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vaccines for western equine encephalitis virus,
eastern equine encephalitis virus, and venezuelan
equine encephalitis virus IE and IIIA variants
- 15 (iii) NUMBER OF SEQUENCES:29
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- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Macintosh 7.5
30 (D) SOFTWARE: Microsoft Word 6.0
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
35 (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
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Application 60/053,652

(B) FILING DATE: July 24, 1997

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

15 (A) LENGTH: 11492 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear

20 (ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

1 ATAGGGCATG GTATAGAGGC ACCTACCCTA CAAACAAATC
GATCCAATAT

25 51 GGAAAGAATT CACGTTGACT TAGACGCTGA CAGCCCATAT
GTCAAGTCGT

101 TACAGCGGAC GTTTCACAA TTTGAGATCG AAGCAAGGCA
GGTCACTGAC

30 151 AATGACCATG CCAATGCCAG AGCGTTTTTCG CATGTGGCAA
CAAAGCTCAT

35 201 TGAGAGCGAA GTCGACCGGG ACCAAGTTAT CTTGGACATT
GGAAGTGCGC

251 CCGTCAGACA TGCACATTC AACCACCGCT ATCATTGTAT
CTGCCCCATG

40 301 ATAAGCGCTG AAGACCCGGA CAGACTACAG CGGTATGCAG
AAAGACTTAA

351 GAGAAGTGAC ATGTACCGAC AAGAATATAG CCTCTNAAGG
CGGCAGACCT

45 401 GCTGGAAGTC ATGTCCACAC CAGACGCAGA GACTCCATCT
CTGTGTATGC

50 451 ACACAGACGC CACGTGTAGG TACTTTGGAA GTGTANGCAG
TATACCAAGA

501 TGTGTACGCA GTCCATGCAC CGACATCAAT CTACCACCAG
GCGCTTAAAG

551 GAGTTAGGAC AATTTACTGG ATAGGTTTTG ACACGACCCC
5 TTTTATGTAC

601 AAAAACATGG CAGGTTCCCTA CCCTACTTAC AACACAACT
GGGCCGACGA

651 GAGAGTATTG GAAGCACGTA ACATTGGCCT CGGTAACTCA
10 GATCTTCAGG

701 AGAGCAGACT TGGAAAACCTT TCAATCCTTA GGAAGAAGAG
GCTCCAACCT
15

751 ACTGATAAGA TCATATTCTC GGTTGGTTCA ACAATCTACA
CAGAGGATAG

801 ATCACTGTTA CGTAGCTGGC ATCTTCCAAA CGTGTTCAC
20 CTGAAAGGAA

851 AGTCTNACTT CACAGGTAGA TGTGGGACCA TTGTCAGCTG
TGAAGGGTAT

901 GTCATCAAAA AGATAACGAT CAGCCCAGGA CTATACGGTA
AAGTTGAGAA

951 CTTGGCGTCC ACGATGCATC GCGAGGGTTT CTTGAGTTGC
AAAGTCACAG
30

1001 ATACGCTGCG CGGCGAGAGG GTTCTTTTGG CTGTGTGTAC
GTATGTACCA

1051 GCCACACTTT GCGATCAGAT GACAGGGAGG GCTCAACCAA
35 CGGATTGTGC

1101 TCAATGGTAG GACGCAAAGA AATACTNACA CAATGCAGAA
CTATCTATTA

1151 CCAGTGGTCG CCCAGGCGTT TTCCAGGTGG GCGCGTGNAC
40 ATCGTGCCGA

1201 CTTGGACGAC GAGAAGGAGC TAGGGGTGCG GGAGCCCACT
CTTACTATGG
45

1251 GCTGCTGCTG GGCTTTCAAG ACCCAGAAAA TTACATCCAT
CTACAAGAAG

1301 CCTGGTACGC AAACAAATTA AGAAAGTACC TGCCGTCTTT
50 TGACTCATT

1351 GTGATTCCGA CGCCTTACCA GCCACGCGGG GGCTCGAATA
TGGGCCTTCC

5 1401 GCCGTNAGGC TCAAGCTGCT GCTTGAACCA ACTGTCAAAC
CCGCACCGGC

1451 TATTACAATG GCCGATGTGG AGCACCTGCG TGGCTTACAG
CAAGAAGCTG

10 1501 AAGAAGTGGC TGCAGCGGGA AGAGATCAGA GAAGCCCTGC
CACCTTGGCT

1551 CCCTGAAATA GAAAAAGAGA CCGTAGAGGC AGAAGTAGAC
CTCATTATGC

15 1601 AAGAGGCAGG AGCAGGTAGC GTGGAGACAC CNACGAGGAC
ATATCAAGGT

1651 AACAAAGTTAC CCAGGTGNAA GAGAAGATTG GGTCTTATCC
CTATACTTTC

20 1701 ACCCCAGGCG GTTTTANAAT NGTNAAAAAC TGGCGTGTAT
CCACCCATTG

25 1751 GCGGAACAAG TACTGGTAAT GACTCACAAA GGCAGGGCCG
GGCGATACAA

1801 AGTCGAGCCA TACCACGGTA NGGTCATTGT ACCCAGAAGG
GACGGCGGGT

30 1851 CCCTGTTCAA GACTTTCAGG CACTGAGTGA GAGCGCCACG
ATCGTTTTCA

1901 ACGAGAGGGA GTTCGTAAAC CAGATATTTT GCACCCACAT
CGCAAGCKTT

35 1951 TCAACTATAG TGAGTCGCTA TTACACTGAC GAAGAGTACT
ATAAGATTGT

40 2001 AAAGACTCAG GACGCAGACT CAGAATACGT CTTTGACATT
GACGCACGAA

2051 AGTGTGTTAA GCGAGAAGAC GCAGGTCCCT TGTGCCTAAC
TGGTGATCTG

45 2101 GTAGATCCAC CATTTCACGA GTTTGCGTAC GAGAGTCTCA
AGACACGACC

2151 AGCAGCACCT CACAAAGTCC CAACCATCGG AGTCTATGGA
50 GTGCCAGGTT

2201 CAGGTAAATC TGGGATCATC AAAAGCGCTG TGACTIONAGAA
AGATCTGGTT

2251 GTGAGTGCAG AGAAGGAAAA CTGCGCAGAA ATTATCAGGG
5 ATGTAAGGAG

2301 GATGAGACGT ATGGATGTTG CTGCTAGGAC TGTGATTCA
GTGCTTCTAA

2351 ATGGGGTTAA GCACCCCGTT AACACCCTGT ACATTGATGA
10 GGCATTTGCC

2401 TGCCATGCAG GGACGCTGCT GGCACCTGATT GCCATCGTCA
AACCTAAGAA
15

2451 AGTGGTATTG TGCGGGGACC CAAAACAATG CGGCTTCTTT
AACATGATGT

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25 GACAAAATCA

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5 3101 ACATTGTGCT GACGAGACAG CAGTGGGAGA CGTTGCACCC
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45 11451 GTTTTTTATT TTGTTTTTAA AATTTCAAAA AAAAAAAAAA
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50

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 11464 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Unknown

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61 TGACATCGAG GAAGATAGTC CCTTCCTCAG AGCATTACAA
CGGAGCTTCC CGCAGTTTGA
121 GGTAGAAGCC AAGCAGGTCA CAGATAATGA CCATGCTAAC
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15 181 GGCATCGAAA TTGATCGAGA CGGAGGTGGA ACCATCCGAT
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241 TGCGCCTGCC CGCAGAATGT ATTCCAAGCA TAAGTACCAT
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301 TGCAGAAGAT CCGGACAGAC TGTTTAAGTA TGCAGCCAAG
20 CTGAAGAAGA ACTGTAAAGA
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25 481 AGTCGCAGTG TATCAGGATG TGTACGCGGT TGACGGACCG
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AGTGGAGTGT GCGGAGCTCT

4141 GTACCGGAAG TACCCCGAAA GCTTCGATTT ACAACCAATA
10 GAAGTGGGGA AAGCTAGATT

4201 GGTCAAAGGT AACTCAAAC ATCTCATTCA TGCAGTGGGG
CCGAATTTTA ACAAAGTGTC

4261 TGAAGTGAA GGTGACAAAC AGCTGGCAGA AGCGTATGAA
TCTATAGCCA GGATTATTAA

15 4321 TGACAACAAT TATAGATCTG TGGCTATTCC GCTTCTGTCC
ACTGGAATAT TTGCCGAAA

4381 CAAGGATAGG TTAATGCAAT CCTTAAACCA TCTGTTAACG
GCATTGGACA CAACAGACGC

4441 AGATGTGGCC ATATACTGCA GAGACAAGAA ATGGGAAGTG
20 ACGTTGAAAG AGGTCGTAGC

4501 CAGGAGAGAG GCGGTAGAGG AGATATGTAT CTCCGAAGAT
TCCTCCGTAG CAGAGCCGGA

4561 TGCAGAGCTG GTTAGAGTTC ACCCTAAGAG CTCTTTGGCT
GGAAGGAAAG GTTACAGCAC

25 4621 TAGCGATGGG AAGACATTCT CATATCTTGA AGGAACCAA
TTTCATCAGG CGGCGAAGGA

4681 CATGGCAGAA ATTAACGCTA TGTGGCCTGC CGCTACAGAG
GCTAATGAGC AGGTGTGCTT

4741 ATACATTCTG GGTGAAAGTA TGAGCAGTAT AAGATCCAAA
30 TGCCCCGTTG AGGAGTCAGA

4801 GGCATCCACC CCACCAAGTA CATTGCCTTG CTTGTGCATC
CACGCTATGA CCCCAGAACG

4861 GGTTCAGCGT TTGAAAGCCT CCCGCCCGA ACAAATTACA
GTTTGTCTT CCTTCCATT

4921 GCCGAAGTAC AGAATAACAG GAGTGCAGAA GATTCAATGT
TCGCATCCTA TACTTTTCTC

4981 TCCTAAAGTA CCTGAGTACA TACACCCTAG AAAGTACCTT
GCAGACGCAG CTTCCGCAA

5 5041 CAATGGGGCA GCCGAATCAA CATCGGTGGA CGTGCAGCCA
CAGCTGGAAG AGAGTCCTGA

5101 GAACACGGAA CAACTGGTGG AGGAGGAAGA CAGTATAAGC
GTGCTGTCTG AGGATACACC

5161 ACACCAAGTG CACCAAGTGG AGGCTGAAGT GCATCGCTTC
10 AGCGCAAGTG CTCAATCTTC

5221 GTCCTGGTCC ATTCCACGTG CATCCGACTT TGATGTCGAG
AGTCTTTCCG TGCTCGAATC

5281 CCTGGGTGCT AATGATACAA TCAGCATGGA GTCGTCCTCA
AACGAAACAG CTCTTGCTTT

15 5341 GCGGACCATT TTTAAGACTC CACCCATTCC AAGGCCTCGA
GTGCAGAGCA CATCCACAGA

5401 CGTGGTTAGT ATCTCAGCAC TCGAGTCTTG TGACAGCACC
AGCGATGCGC GTAGCATAGA

5461 CTCGGATGAA ACCGATGTTT CCATCTTTGA CAAAAGGTTG
20 GAGTTCCTGG CCAGACCTGT

5521 TCCCGCACCG CGAACCAAAT TTAGGACTCC ACCCGTCCCG
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5581 ATTACATCCT TTGTCTAGTA GATCAAGCTC GCGCTCTAGC
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25 5641 TGTTAACCGA GTGATCACTA GGAAGAATT TGAGTCCTTC
GTTGCCCAAC AGCAATGACG

5701 GTTCGACGCG GGCGGTACA TTTTCTCCTC GGATACTGGT
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5761 ATCAGTAAGG CAGACAGTAT TGTCTGAAGT GGTGCTAGAG
30 AGGACTGAGT TAGAGATCTC

5821 GTACGCCCCG CGCCTCGACC TGAACAAAGA AGAAATACTG
AGAAAGAAGT TACAACAGAA

5881 CCCTACGCAA GCTAACCGGA GTAGATATCA GTCACGGAGG
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5941 AACAACTAAG AGAATCTTAC AGGGATTAGG TCACTACCTG
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 AGTGTGAACA GAGCGTTCTC
 5 6061 CAGTCCCAA GTTGCTGTTG AAGCATGTAA CGTTGTTCTG
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 6181 ATGTTGCTTG GATACGGCGA GTTTTTGCC TCCAAGTTG
 10 CGTAGCTTTC CGAAGAAACA
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 GCAATTCAGA ACACGCTGCA
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 15 6361 TGTACTGGAT TCTGCGGCCT TCAATGTAGA GTGTTTTAAA
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 GAGGAAAATG TGGTCAACTA
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 20 GCAAAGACTC ATAATTTAGA
 6541 CATGCTGCAA GACATACCCA TGGACAGGTT TATTATGGAT
 TTAAAAGAG ATGTCAAGGT
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 CAAGTAATCC AGGCTGCAGA
 25 6661 TCCATTGGCT ACAGCATACC TATGTGGGAT TCATAGAGAA
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 6721 AGTTCCTGTTG CCAACATAC ACACATTATT TGACATGTCT
 GCTGAGGATT TCGACGCCAT
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 30 ACAGACATAG CGTCATTGCA
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5 7081 AAAC TCCCCT TGC GCCCGT TCATTGGCGA CGACAATATC
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7141 CAAACTCATG GCCGATAGGT GCGCTACATG GTTGAACATG
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7201 AGTGGTTGGC GAGAAAGCTC CCTACTTCTG TGGTGGGTTT
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7261 CGGAACTGCA TGCCGTGTAG CAGACCCTTT GAAGAGATTA
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7321 GGCTGTGGAT GATGAACATG ATGATGACAG GCGTCGAGCA
CTACAGGAGG AATCTGCCCG
15 7381 GTGGAACCGG GTGGGAATTT TTTCCGAGCT GTGCAAAGCC
GTCGAGTCGC GATATGAAAC
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7501 CAGTTGTCTA AGAGGGGCTT CTATATCCCT CTACGGCTAA
20 CCTGAATGGA CTGCGACGTA
7561 GTCAAGTCCG CCGAAATGTT TCCTTATCAA CCAATGTACC
CAATGCAGCC CATGCCCTTC
7621 CGCAACCCTT TTGCGACTCC CAGAAGACCA TGTTTCCAA
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25 7681 ATGCAGGTGC AAGAGCTGGC AAGGTCCATG GCCAACTTGA
CGTTCAAGCA ACGGCGAGAT
7741 GTGCCGCCCG AGGGTCCACC GGCTAAGAAG AAGAAGAAGG
ACAAC TCGCA ACAAGGTGGT
7801 CGGAATCAGA ATGGAAAGAA AAAGAACAAG CTAGTAAAGA
30 AAAAGAAGAA GACAGGGCCA
7861 CCACCCCAA AAAATACTGG TGGCAAAAAG AAAGTCAATA
GGAAGCCAGG GAAGAGACAA
7921 CGAATGGTTA TGAAGTTGGA GTCAGACAAG ACATTCCCTA
TCATGCTAGA TGGAAAAGTT

7981 AATGGATATG CATGCGTGGT CGGTGGCAAG CTGTTTAGAC
CACTGCATGT GGAGGGTAAG
8041 ATTGACAATG ACGTGTGTC CTCCTCAAG ACCAAAAGG
CATCTAAGTA TGATCTGGAG
5 8101 TATGCTGATG TGCCGCAGAG CATGCGCGCA GACACATTTA
AATACACTCA TGAAAAACCC
8161 CAGGGCTATT ACAGCTGGCA CCATGGAGCA GTACAGTATG
AAAATGGCAG ATTCACAGTG
8221 CCCAAAGGAG TCGGAGCCAA AGGAGATAGC GGTGCCCCA
10 TACTTGACAA CCAAGGGCGT
8281 GTGGTCGCTA TTGTGCTTGG CGGAGTGAAT GAAGGCTCCA
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8341 ACGTGAACG AAAAAGGGT TACAGTCAA TACACCCCCG
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15 8401 CTGGTGACCA CCATGTGCCT GCTAGCCAAT GTCACGTTCC
CGTGCAACCA ACCACCCATC
8461 TGCTACGACC GTAAGCCAGC AGAGACTTTG TCCATGCTCA
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8581 GAGGAGCTGT TTAAGGAGTA CAAGTTAACA CGCCCGTACA
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8641 GCGGTCGGAA GTTGCCACAG CCCCATAGCC ATAGAGGCGG
TAAGGAGCGA AGGGCACGAT
25 8701 GGCTATGTAC GACTCCAGAC CTCATCTCAG TATGGATTAG
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8821 TCTGTTTATA CCTCACGTCC TTGCCACATA ATAGATGGGC
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GAGAACTGTA CACACACCA

9001 CCAGAGCACG GAGCTGAACA ACCTTGTCAC GTGTACGCTC
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9061 GCTTACGTGG AAATGCACCT TCCAGGATCC GAAGTGGACA
GTACTTTACT GTCCATGAGC
5 9121 GGTAGTTCTG TTCATGTGAC TCCACCTGCC GGGCAAAGCG
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9181 GGTGGCACCA AGATCTCTGA AACCATCAAT TCAGCTAAAC
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9241 ACATCTCAGT GCAGGGCATA CCGTACACAG AATGACAAAT
10 GGGTGTACAA TTCGGATAAA
9301 CTGCCTAAAG CATCGGGAGA AACTCTCAA GCAAATTGC
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TCACCTTTGG GTTCCGCTCT
15 9421 GTGTCTCTGA AACTTCATCC TAAGAACCCC ACCTTCCTAA
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9481 GAACCAGCTT ACACCCACGA ACTTATAACC CACCCTGTGG
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20 AAAGGTACTG GTCTCAAGAA
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CGATAGTGAC GACATCCATT
25 9721 GCGGCATCCG TATGGCTGTT TTGCAAATCA CGGATTTTCAT
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9781 ACTCCGAATG CCAGCATGCC TCTGTGCTTA GCCGTCTTGT
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9841 GCCGAAACTA CTTGGGAATC CCTAGATCAC CTCTGGAACC
30 ACAACCAGCA GATGTTCTGG
9901 AGTCAGCTGC TAATCCCGCT AGCAGCACTG ATAGTTGCTA
CCCGCTTGCT GAAATGTGTG
9961 TGTTGCGTTG TGCCTTTTTT AGTCGTGGCC GCGCCGCTAG
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10021 GCGACTACGA TGCCGAACCA AGTGGGGATC CCGTATAATA
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10081 TATGCACCTC TACCTATTAG CATAGTACCT ACTAAAGTGA
AGCTGATTCC AACAGTGAAT

5 10141 CTTGAGTACA TTACATGCCA TTACAAGACT GGAATGGATT
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10201 GGCACTCAGG AGTGTTCTCC AACTTACAGG CCGGACGAGC
AATGCAAAGT CTTCTCTGGA

10261 GTATACCCAT TTATGTGGGG AGGGGCGTAT TGCTTTTGGC
10 ATACGGAGAA TACCCAGATA

10321 AGCAAGGCGT ACGTGACGAA ATCGGAAGAT TGCCTCACCG
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10381 GCACATACAG CCTCAGTCCA AGCCTTCTTA AATATTACAG
TTGGAGGACA CTCAACGACA

15 10441 GCAGTGGTGT ATGTGAATGG AGAGACTCCC GTTAATTTTA
ATGGAGTGAA GCTGACCGCG

10501 GGCCCTCTGT CCACAGCCTG GTCGCCGTC GACAAGAAGA
TCGTGCAGTA CGCCGGGGAA

10561 ATTTATAACT ATGACTTTCC GGAATATGGA GCCGGCCACG
20 CAGGAGCGTT TGGTGACATC

10621 CAGGCTAGGA CGGTATCTAG TTCCGATGTA TACGCCAACA
CAAACCTTGT GCTGCAGAGA

10681 CCCAAAGCCG GAGCGATCCA TGTCCCGTAC ACCCAGGCC
CATCTGGGTA TGAACAATGG

25 10741 AAGAAAGATA AACCACCATC CCTCAAGTTC ACAGCCCCGT
TCGGTTGTGA AATTTACACC

10801 AACCCATCC GTGCTGAAAA CTGCGCTGTG GGATCAATTC
CGCTAGCTTT TGACATTCCC

10861 GATGCTCTGT TTACCAGGGT GTCCGAAACA CCGACATTAT
30 CTGCTGCCGA GTGCACTCTG

10921 AACGAGTGTG TATATTCATC CGACTTTGGC GGGATCGCTA
CAGTCAAATA CTCGGCGAGC

10981 AAGTCAGGCA AATGTGCAGT TCATGTACCC TCAGGCACGG
CTACATTGAA AGAAGCCGCA

11041 GTCGAGTTGG CCGAACAGGG TTCGGCTACT ATACATTTTT
 CGACTGCCAG CATTTCATCCG
 11101 GAGTTTAGAC TCCAGATATG CACGTCTTAC GTTACGTGCA
 AAGGGGATTG TCACCCTCCG
 5 11161 AAAGATCACA TTGTGACGCA TCCCAATAC CACGCCAGT
 CATTTACAGC TCGGGTATCA
 11221 AAAACCGCTT GGACGTGGTT AACATCCTTA CTGGGAGGGT
 CAGCTATAAT TATAATAATT
 11281 GGACTTGTGT TAGCCACAGT TGTGGCTATG TATGTGCTGA
 10 CCAACCAGAA ACATAATTAG
 11341 TATTAGCAGC GATTGGCATG CTGCTTGTAAG AGTTTTATTA
 CAAATAACGT GCGGCAATTG
 11401 GCGAGCCGCT TTAATTAGAA TTTTATTTTC TTTTACCATA
 ATTGGATTTT GTTTTTAATA
 15 11461 TTTC

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 4003 base pairs
 (B) TYPE: Nucleic acid
 (C) STRANDEDNESS: Double
 (D) TOPOLOGY: Unknown
 Nucleotide Sequence of the BeAn8 26S Subgenomic
 25 cDNA containing last portion of NS4, the 26S promoter,
 the complete BeAn8 structural genes and a portion of
 the 3' noncoding region.
 30
 1 CCCAGTAACT CTCTACGGCT GACCTGAATG GACTGTAACG
 TAGTTCAGTC
 51 CGCAACCATG TTCCCTTACC AATCACCAAT GTTTCCAATG
 35 CAACCAGCGC
 101 CTTTTCGCAA CCCGTACGCT CCTCCTAGAA GACCGTGGTT
 CCCTAGAACC
 40 151 GATCCCTTCT TAGCCATGCA GGTGCAGGAG TTGGCCCGAT
 CAATGGCGAG
 201 CTTGACGTTT AAACAGCGTC GAGATACGCC ACCCGAGGGG
 CCACCTGCTA

251 AGAAGAAGCG TAAGGAGCCT CAACAGCAGG TAGCTCAGGC
GCAGGTTAAG

5 301 AAAAAGAACG GAAAACCGAA GAAGAAGAAA AGTAACGGAG
CACCACCCCC

351 AAAAAATCAG AAGAGCACCA AGAAGAAGAC CAATAAGAAA
CCTGGAAAAA

10 401 GACAACGGAT GGTATGAAG TTAGAATCAG ACAAACATT
TCCTATTTTG

451 CTGGATGGAA AAATTAATGG ATATGCTTGC GTTGTTGGAG
15 GAAAGCTATT

501 TCGACCCATG CACGTGGAAG GCAAAATTGA CAATGAACT
CTTGCCCTCCC

20 551 TGAAGACGAA GAAGGCATCC AAATACGACC TAGAGTATGC
CGATGTTCCCT

601 CAAAGCATGC GAGCAGATAC CTTTAAATAC ACACATGATA
AGCCCAAGG

25 651 GTATTATAAT TGGCATCACG GCGCCGTGCA GTATGAAAAT
GGGAGATTCA

701 CGGTGCCGAA AGGTGTGGGA GCGAAAGGAG ACAGTGGACG
30 CCCCATACTA

751 GATAATCAAG GCAGAGTCGT GGCCATTGTG CTGGGAGGGG
TGAATGAAGG

35 801 ATCCAAGACA GCTTTGTCCG TAGTTATGTG GAATGAAAAA
GGGGTCACCG

851 TAAAATATAC ACCAGAAAAC TGTGAGCAAT GGTCCTAGT
TACCACCATG

40 901 TGTCTTCTCG CCGATGTTAC GTTCCCTTGT TCCACTCCAC
CAATTTGCTA

951 CGACCGAGCA CCCGCAGAAA CCCTGATGAT GCTTAGCAAG
45 AACATTGACA

1001 ATCCTGGCTA TGATGAATTG CTGGAAGCAG TGCTGAAATG
CCCCGGCAGA

50 1051 CAGAAGAGAT CTACGGAGGA GTTATTTAAG GAGTACAAAC
TTACACGTCC

1101 GTACATGGCC AAGTGTGTGC GGTGTGCCGT TGGAAGTTGC
CACAGCCCGA

5 1151 TCGCTATAGA AGCAGTAAGA AGCGACGGGC ATGACGGCTA
CATCCGAATA

1201 CAGACATCAT CACAGTACGG TTTAGACCCC TCCGGGAACG
TTAAGAGCAG

10 1251 AGTTATGAGG TATAATCTGT ATGGCAAGAT CGTAGAAGTT
CCATTACATC

1301 AGGTTTCATT ACACACATCT CGGCCTTGCC ACATTATTGA
TGGTCACGGA

15 1351 TATTTCTCC TCGCACGCTG CCCAGAGGGC GACTCTATCA
CCATGGAGTT

1401 TAAGAAGGAT TCCGTCACCC ATTCCTGTTC AGTGCCTTAT
20 GAAGTGAAAT

1451 TCACACCCGT GGGCAGAGAA TTATATAGCC ATCCCCAGA
ACACGGCACA

25 1501 GAACATCCGT GCCGTGTGTA TGCCCACGAC GCCCAGCAAA
AAGATGCGTA

1551 TGTGGAGATG CACCTGCCCCG GGTCCGAAGT TGACAGTTCC
30 CTGCTCTCCA

1601 TGAGCGGTAG TGCGGTCCGG GTAACACCAC CATCAGGGCA
AAGTGTCCTT

1651 GTGGAGTGCA ACTGTGGCTC CGCTGTGTCG GAAACCATAA
35 AACTGCAAA

1701 ATCATAACAGC CAATGCACAA AAACATCACA ATGCCGCGCG
TACCGTCTGC

40 1751 AGAATGATAA GTGGGTATAC AATTCAGACA AACTTCCAAA
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1801 GAAACGCTGA AAGGGAACT GCATGTACCT TATCTCCTTT
45 CCGAAGCGAA

1851 GTGTACCGTA CCTTTAGCAC CCGAGCCAAT AGTAACCTTC
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1901 TCGTATCTTT GAAATTGCAT CCACGGAATC CGACATATTT
50 GACTACCCGC

1951 CAGCTAGATG GAGAACCGAA TTACACCCAC GAGCTAATTT
CAGAGCCAAC

2001 AACTAGAAAT TTTACAGTGA CTGAGCATGG ATGGGGATAC
5 GTTTGGGGTA

2051 ATCACCCGCC TCAGAGGTAC TGGGCACAGG AGACAGCTCC
AGGCAACCCG

10 2101 CATGGGCTGC CGCACGAGGT GATTACTCAT TACTATAACA
GGTACCCAAT

2151 GTCCACGATT TTCGGACTAT CGATTTGCGC CGCAGTGGTA
15 ACCACCTCAA

2201 TAGCCGCATC CACCTGGCTG TTGTGCAAGT CGAGAGTATC
TTGTTTACT

2251 CCGTATCGAC TGACCCCGAA TGCTCAGTTA CCTGTGTGTC
20 TAGCCTTCCT

2301 GTGCTGCGCG AGGACAGCCC GTGCAGAGAC CACATGGGAA
TCACTAGACC

25 2351 ATTTATGGAA CCATAATCAA CAGATGTTCT GGAGTCAACT
GCTCATTCCT

2401 CTAGCCGCGC TCATTGTGGT GACCCGCTTG CTGAAGTGCA
30 TGTGTTGCGT

2451 CGTTCCTTTT TTAGTCCTAG CAGGCGCCGC AAGCGTCGGC
GCCTACGAAC

2501 ACGCAACCAC GATGCCGAAT CAGGTGGGGA TCCCGTATAA
35 TACAGTAGTC

2551 AACCGCGCAG GTGACGCACC ATTGGCAATC AGCATTATTC
CAACCAAGAT

40 2601 ACGGCTAATT CCTACTTTGA ATTTAGAATA TATTACATGC
CACTATAAGA

2651 CAGGATTAGA TTCACCTTTC ATTAAATGCT GCGGAACGCA
45 GGAATGCCCC

2701 CAAGTGAATA GACCCGATGA ACAGTGTAAT GTCTTCACGG
GGGTGTATCC

2751 GTTTATGTGG GGAGGCGCCT ACTGCTTCTG TGACTIONGAA
50 AACACGCAAA

2801 TTAGTCGAGC GTATGTGATG AAATCAGATG ACTGCTCAGC
TGACCACGCC

2851 TTGGCCTACA AAGCTCATA CAGCTCAGTC CAAGCTTTTC
5 TGAATATAAC

2901 TGTGGGAGAG CAATCGACGA CAGCGGTAGT GTACGTGAAT
GGAGAAACAC

2951 CGGTCAATTT TAACGGCATT AAATGGTGTG CAGGCCCTTT
10 ATCAACTGCC

3001 TGGACCCCAT TTGATCGGAA AGTGGTGCAG TACGCCGGAG
AGATCTACAA
15

3051 TTATGACTTC CCGGAGTACG GAGCTGGGCA TGCAGGGGCG
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3101 TTCAAGCCAG AACAATCACC AGTAATGACC TGTACGCCAA
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3151 GTATTGCAA GACCCAACAC AGGCACCATC CATGTTCTT
ACACGCAGGC

3201 ACCGTCAGGC TTTGAGCAGT GGAAGAAAGA CAAACCACCA
25 TCATTAAAGT

3251 ACACCGCACC ATTTGGGTGC GAAATTCATG TGAATCCCGT
CAGAGCTGAG
30

3301 AATTGCGCAG TAGGATTTAT ACCATTAGCC TTCGACATAC
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3351 GTTTACCAGG GTGTCAGAAA CACCGACGTT GTCGAGCGCT
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3551 CCCTTCACTT TTCGACCGCC AGTATACACC CAGAGTTTAA
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3651 TATTGTGACG CACCCCAGC ACCACGCCA GACATTTACA
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5 3701 CCAAGACCGC TTGGACGTGG TTAACGTCAC TCTTAGGAGG
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3751 ATTATTATAA TTGGCCTTGT ATTAGCAACT GTTGTGCTA
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10 3801 GACCAACCAG AACATAATT AGACCACAGC AGCGATTGGA
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3851 TTAGAAACAT GTAGCGGCAA TTGGCAAGCC GCCTATAAAT
GTTTAGCAGC

15 3901 AATTGGCAAG CTGCATATAT AAATTACCTA GCGGCAATTG
GCACGCCGCT

3951 TATAAAATTT TTATTTTCTT TTACCAATAA TTGGATTTTG
20 TTTTAAATAT

4001 TTC

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What is claimed is:

1. A DNA comprising an isolated and purified western equine encephalitis (WEE) virus cDNA fragment
5 coding for infectious western equine Encephalitis virus genome.
2. A DNA according to claim 1 wherein said
10 WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.
3. The DNA according to claim 1, wherein
said cDNA fragment contains a deletion in the E3-E2
15 cleavage site.
4. The DNA according to claim 3, wherein
said cDNA fragment deletion is 5 amino acids at the
E2-E2 cleavage site said amino acids being Arg Arg Pro
20 Lys Arg.
5. The DNA according to claim 4 wherein said
cDNA fragment further contains a suppressor mutation.
6. The DNA according to claim 5 wherein said
25 suppressor mutation is a substitution of glutamic acid at codon 182 of E2 to lysine.
7. The DNA according to claim 5 wherein said
suppressor mutation is a substitution of glutamic acid
30 at codon 181 of E2 to lysine.
8. The DNA according to claim 2 wherein said
promoter is T7 promoter of pBluescript KS and said DNA
is pWE2000.

9. An infectious WEE virus RNA transcript encoded by the cDNA fragment of claim 1.

5 10. A DNA according to claim 5 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

10 11. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 5.

15 12. A DNA according to claim 6 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

15 13. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 6.

20 14. A DNA according to claim 7 wherein said WEE cDNA fragment is operably linked to a promoter such that said cDNA is transcribed.

25 15. An attenuated WEE virus RNA transcript encoded by the cDNA fragment of claim 7.

 16. Infectious WEE virus particles containing an RNA transcript according to claim 9.

30 17. Attenuated WEE virus particles containing an RNA transcript according to claim 11.

 18. Attenuated WEE virus particles containing an RNA transcript according to claim 13.

19. Attenuated WEE virus particles
containing an RNA transcript according to claim 15.

20. A live attenuated western equine
5 encephalitis (WEE) virus vaccine comprising attenuated
western equine encephalitis virus according to claim
17.

21. A live attenuated western equine
10 encephalitis (WEE) virus vaccine comprising attenuated
Western Equine Encephalitis virus according to claim
18.

22. A live attenuated western equine
15 encephalitis (WEE) virus vaccine comprising attenuated
western equine encephalitis virus according to claim
19.

23. A DNA comprising an isolated and
20 purified venezuelan equine encephalitis virus IE
variant (VEE IE) cDNA fragment coding for infectious
venezuelan equine encephalitis virus IE variant virus
genome.

25 24. A DNA according to claim 23, wherein
said cDNA fragment has the sequence of SEQ ID NO:2 or
a portion thereof, or an allelic portion thereof.

30 25. The DNA according to claim 23, wherein
said cDNA fragment contains a deletion in the E3-E2
cleavage site.

26. A DNA according to claim 25, wherein
said cDNA fragment contains a deletion according to

claim 25 wherein said deletion is four amino acids
said amino acids being Arg Gly Lys Arg.

27. A DNA fragment according to claim 26
5 wherein said VEE IE cDNA further contains a suppressor
mutation.

28. A DNA according to claim 23 wherein said
VEE IE cDNA fragment is operably linked to a promoter
10 such that said cDNA is transcribed.

29. An infectious VEE IE virus RNA
transcript encoded by the cDNA fragment of claim 23.

15 30. A DNA according to claim 27 wherein said
VEE IE cDNA fragment is operably linked to a promoter
such that said cDNA is transcribed.

20 31. An attenuated VEE IE virus RNA
transcript encoded by the cDNA fragment of claim 27.

32. Infectious VEE IE virus particles
containing an RNA transcript according to claim 29.

25 33. Attenuated VEE IE virus particles
containing an RNA transcript according to claim 31.

30 34. A live attenuated venezuelan equine
encephalitis virus IE variant (VEE IE) virus vaccine
comprising attenuated venezuelan equine encephalitis
virus IE variant virus according to claim 33.

35 35. A bivalent alphavirus vaccine comprising
live attenuated western equine encephalitis (WEE)
virus comprising an attenuating mutation and live

attenuated venezuelan equine encephalitis virus IE variant (VEE IE) comprising an attenuating mutation.

36. A bivalent alphavirus vaccine according
5 to claim 35 wherein said live attenuated western
equine encephalitis virus is chosen from the group
consisting of attenuated WEE comprising a deletion in
the E3-E2 cleavage site and a substitution of glutamic
acid at codon 182 of E2 to lysine, and attenuated WEE
10 comprising a deletion in the E3-E2 cleavage site and a
substitution of glutamic acid at codon 181 of E2 to
lysine.

37. An attenuated western equine
15 encephalitis virus wherein said virus comprises an
attenuating mutation selected from the group
consisting of: a C to T change at nucleotide 7 of the
5' noncoding region of the WEE genome, a A to G change
at nucleotide 13 of the 5' noncoding region of the WEE
20 genome, a T to A change at nucleotide 25 of the 5'
noncoding region of the WEE genome, and a deletion of
an A at nucleotide 22 of the 5' noncoding region of
the WEE genome.

25 38. A pharmaceutical formulation comprising
attenuated WEE virus particles according to claim 17
in an effective immunogenic amount in a
pharmaceutically acceptable carrier.

30 39. A pharmaceutical formulation comprising
attenuated WEE virus particles according to claim 18
in an effective immunogenic amount in a
pharmaceutically acceptable carrier.

40. A pharmaceutical formulation comprising attenuated WEE virus particles according to claim 19 in an effective immunogenic amount in a pharmaceutically acceptable carrier.

5

41. A pharmaceutical formulation comprising attenuated VEE IE virus particles according to claim 33 in an effective immunogenic amount in a pharmaceutically acceptable carrier

10

42. An attenuated chimeric virus comprising nonstructural sequences from a first alphavirus and structural sequences from a second alphavirus resulting in attenuation of the second alphavirus.

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43. An attenuated chimeric virus according to claim 42 wherein wherein said first alphavirus is western equine encephalitis virus and said second alphavirus is chosen from the group consisting of:
Sindbis virus, Aura virus, Barmah Forest virus, Bebaru Cabassou virus, Chikungunya virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylogach virus, Mayaro virus, Middelburg virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Ross River virus, Sagiyama virus, Semliki Forest virus, SAAR87 virus, Tonate virus, Una virus, venezuelan equine encephalitis virus, and Whataroa virus.

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44. An attenuated second virus according to claim 43 wherein said second alphavirus is eastern equine encephalitis virus.

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45. An attenuated chimeric second virus according to claim 42 wherein said first alphavirus is

Venezuelan equine encephalitis virus variant IE and said second alphavirus is chosen from the group consisting of: venezuelan equine encephalitis virus, western eqine encephalitis virus, eastern equine
5 encephalitis virus, Sindbis virus, Aura virus, Barmah Forest virus, Bebaru Cabassou virus, Chikungunya virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Middelburg virus, Mucambo virus, Ndumu virus,
10 O'nyong-nyong virus, Pixuna virus, Ross River virus, Sagiyama virus, Semliki Forest virus, SAAR87 virus, Tonate virus, Una virus and Whataroa virus.

46. An attenuated chimeric second virus
15 according to claim 44 wherein said second virus is venezuelan equine encephalitis virus variant IA.

47. An attenuated chimeric second virus according to claim 42 wherein said first alphavirus is
20 venezuelan equine encephalitis virus variant IA and said second alphavirus is chosen from the group consisting of: Venezuelan equine encephalitis virus, western eqine encephalitis virus, eastern equine encephalitis virus, Sindbis virus, Aura virus, Barmah
25 Forest virus, Bebaru Cabassou virus, Chikungunya virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Middelburg virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Ross River virus,
30 Sagiyama virus, Semliki Forest virus, SAAR87 virus, Tonate virus, Una virus, and Whataroa virus.

48. An attenuated chimeric second virus according to claim 47 wherein said second virus is
35 venezuelan equine encephalitis virus variant IE.

49. An attenuated chimeric second virus according to claim 47 wherein said second virus is Venezuelan Equine Encephalitis virus variant IIIA.

5

50. An attenuated virus vaccine comprising chimeric virus according to claim 42 wherein said vaccine is directed against said second alphavirus.

10 51. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 17 wherein said attenuated WEE is inactivated.

15 52. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 18 wherein said attenuated WEE is inactivated.

20 53. An inactivated western equine encephalitis virus (WEE) vaccine comprising attenuated WEE according to claim 19 wherein said attenuated WEE is further inactivated.

25 54. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 33 wherein said attenuated VEE IE is further inactivated.

30 55. An inactivated eastern equine encephalitis virus (EEE) vaccine comprising attenuated EEE according to claim 44 wherein said attenuated virus is further inactivated.

56. An inactivated venezuelan equine encephalitis virus variant IA (VEE/IA) vaccine comprising attenuated VEE/IA according to claim 46 wherein said attenuated virus is further inactivated.

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57. An inactivated venezuelan equine encephalitis virus variant IIIA (VEE/IA) vaccine comprising attenuated VEE IIIA according to claim 49 wherein said attenuated virus is further inactivated.

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58. An inactivated venezuelan equine encephalitis virus variant IE (VEE IE) vaccine comprising attenuated VEE IE according to claim 48 wherein said attenuated virus is further inactivated.

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59. A method for expressing a protein said method comprising cloning a gene encoding said protein into a an attenuated virus replicon, said replicon chosen from the group consisting essentially of attenuated WEE and attenuated VEE IE wherein transcription of said replicon yields RNA capable of infecting a cell in which said protein is to be expressed.

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60. A method for the diagnosis of western equine encephalitis virus (WEE) infection comprising the steps of :

25

(i) contacting a sample from an individual suspected of having a WEE infection with all or a unique portion of WEE; and

30

(ii) detecting the presence or absence of a WEE infection by detecting the presence or absence of a complex formed between WEE and antibodies specific therefor in the sample.

61. A method for the diagnosis of venezuelan equine encephalitis virus variant IE (VEE IE) infection comprising the steps of :

- 5 (i) contacting a sample from an individual suspected of having a VEE IE infection with all or a unique portion of VEE IE; and
- (ii) detecting the presence or absence of a VEE IE infection by detecting the presence or absence
10 of a complex formed between VEE IE and antibodies specific therefor in the sample.

62. A method for the diagnosis of western equine encephalitis virus (WEE) from a sample using
15 the polymerase chain reaction, said method comprising:

- (i) extracting RNA from the sample;
- (ii) reverse transcribing the RNA of (i) to DNA;
- (iii) contacting said DNA with
20 (a) at least four nucleotide triphosphates,
(b) a primer that hybridizes to WEE DNA, and
(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme,
25 whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

(iv) denaturing said duplex to release said first DNA product from said DNA;

30 (v) contacting said first DNA product with a reaction mixture comprising:

- (a) at least four nucleotide triphosphates,
(b) a second primer that hybridizes to said first DNA, and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

(vi) denaturing said second DNA product from said first DNA product;

(vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

(viii) fractionating said first and second DNA products generated from said WEE DNA; and

(ix) detecting said fractionated products for the presence or absence of WEE in a sample.

63. A method for the diagnosis of venezuelan equine encephalitis variant IE virus (VEE IE) from a sample using the polymerase chain reaction, said method comprising:

(i) extracting RNA from the sample;

(ii) reverse transcribing said RNA of (i) to DNA;

(iii) contacting said DNA with

(a) at least four nucleotide triphosphates,

(b) a primer that hybridizes to VEE IE DNA,

and

(c) an enzyme with polynucleotide synthetic activity,

under conditions suitable for the hybridization and extension of said first primer by said enzyme, whereby a first DNA product is synthesized with said DNA as a template therefor, such that a duplex molecule is formed;

(iv) denaturing said duplex to release said first DNA product from said DNA;

(v) contacting said first DNA product with a reaction mixture comprising:

- 5 (a) at least four nucleotide triphosphates,
 (b) a second primer that hybridizes to said first DNA, and
 (c) an enzyme with polynucleotide synthetic activity,

10 under conditions suitable for the hybridization and extension of said second primer by said enzyme, whereby a second DNA product is synthesized with said first DNA as a template therefor, such that a duplex molecule is formed;

15 (vi) denaturing said second DNA product from said first DNA product;

 (vii) repeating steps iii-vi for a sufficient number of times to achieve linear production of said first and second DNA products;

20 (viii) fractionating said first and second DNA products generated from said VEE IE DNA; and

 (ix) detecting said fractionated products for the presence or absence of VEE IE in a sample.

25 64. A method for providing protective immunity against a second alphavirus to individuals with pre-existing immunity to a first alphavirus said method comprising administering to said individuals an effective amount of live attenuated second alphavirus.

30 65. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second alphavirus is attenuated western equine encephalitis
35 virus.

66. The method of claim 65 wherein said western equine encephalitis (WEE) virus is chosen from the group consisting of: attenuated WEE having a substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a substitution of glutamic acid at codon 181 of E2 to lysine, attenuated WEE having a C to T change at nucleotide 7 of the 5' noncoding region of the WEE genome, attenuated WEE having a A to G change at nucleotide 13 of the 5' noncoding region of the WEE genome, attenuated WEE having a T to A change at nucleotide 25 of the 5' noncoding region of the WEE genome, and attenuated WEE having a deletion of an A at nucleotide 22 of the 5' noncoding region of the WEE genome.

67. A method for providing protective immunity against a second alphavirus according to claim 64, wherein said live attenuated second alphavirus is attenuated venezuelan equine encephalitis virus variant IE.

68. A WEE infection diagnostic kit comprising primers specific for WEE and ancillary reagents suitable for use in detecting the presence or absence of WEE in a mammalian sample.

69. A VEE IE infection diagnostic kit comprising primers specific for VEE IE and ancillary reagents suitable for use in detecting the presence or absence of VEE IE in a mammalian sample.

70. An isolated and purified Venezuelan equine encephalitis variant IIIA cDNA encoding the VEE IIIA structural genes.

71. An amino acid fragment encoded by the cDNA fragment according to claim 70.

5 72. A polypeptide encoded by the amino acid fragment according to claim 71.

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Assembly module WE3'-17 1 / 7

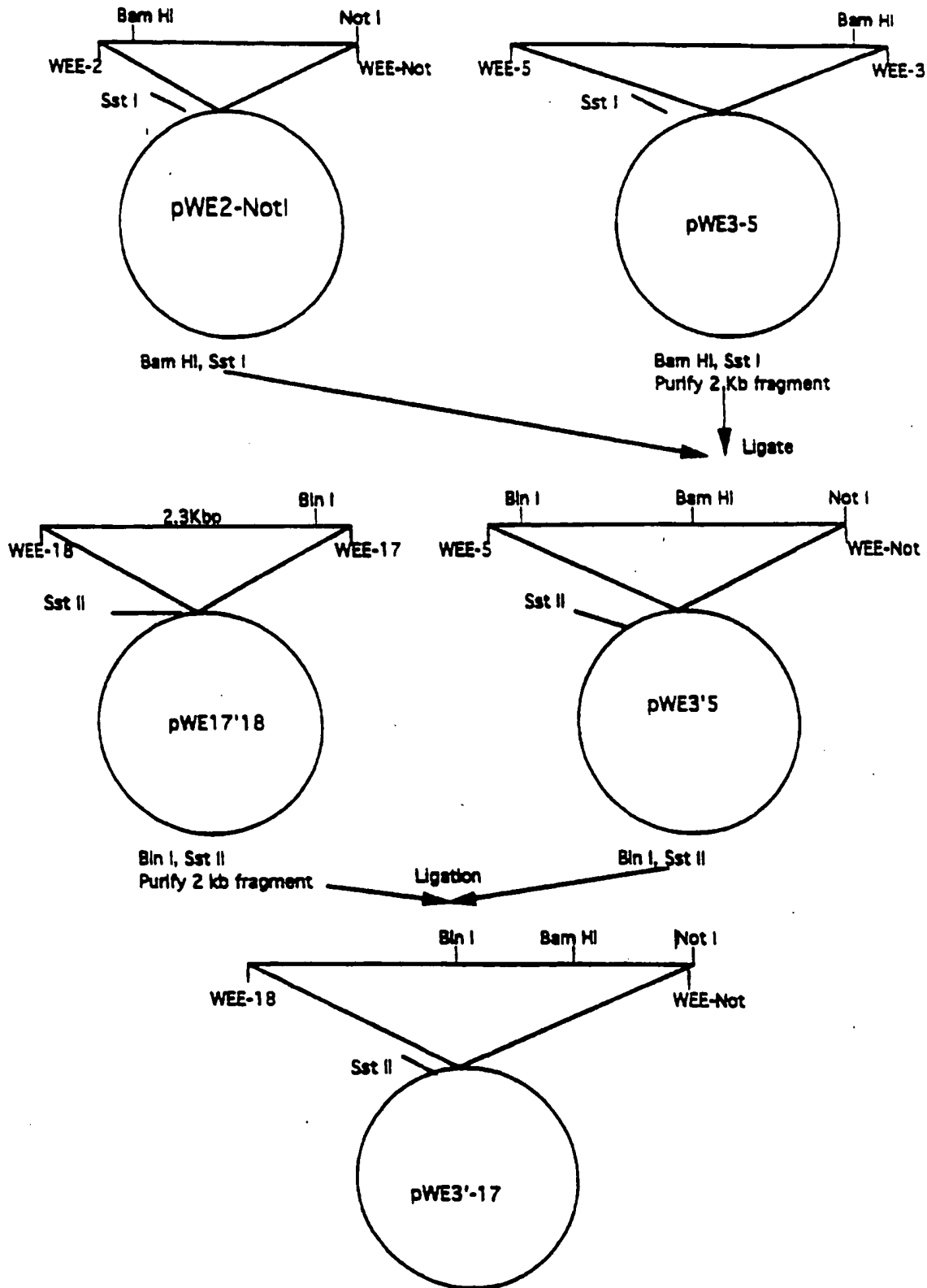


FIGURE 1A

Assembly c noc J pWES'-18 2 / 7

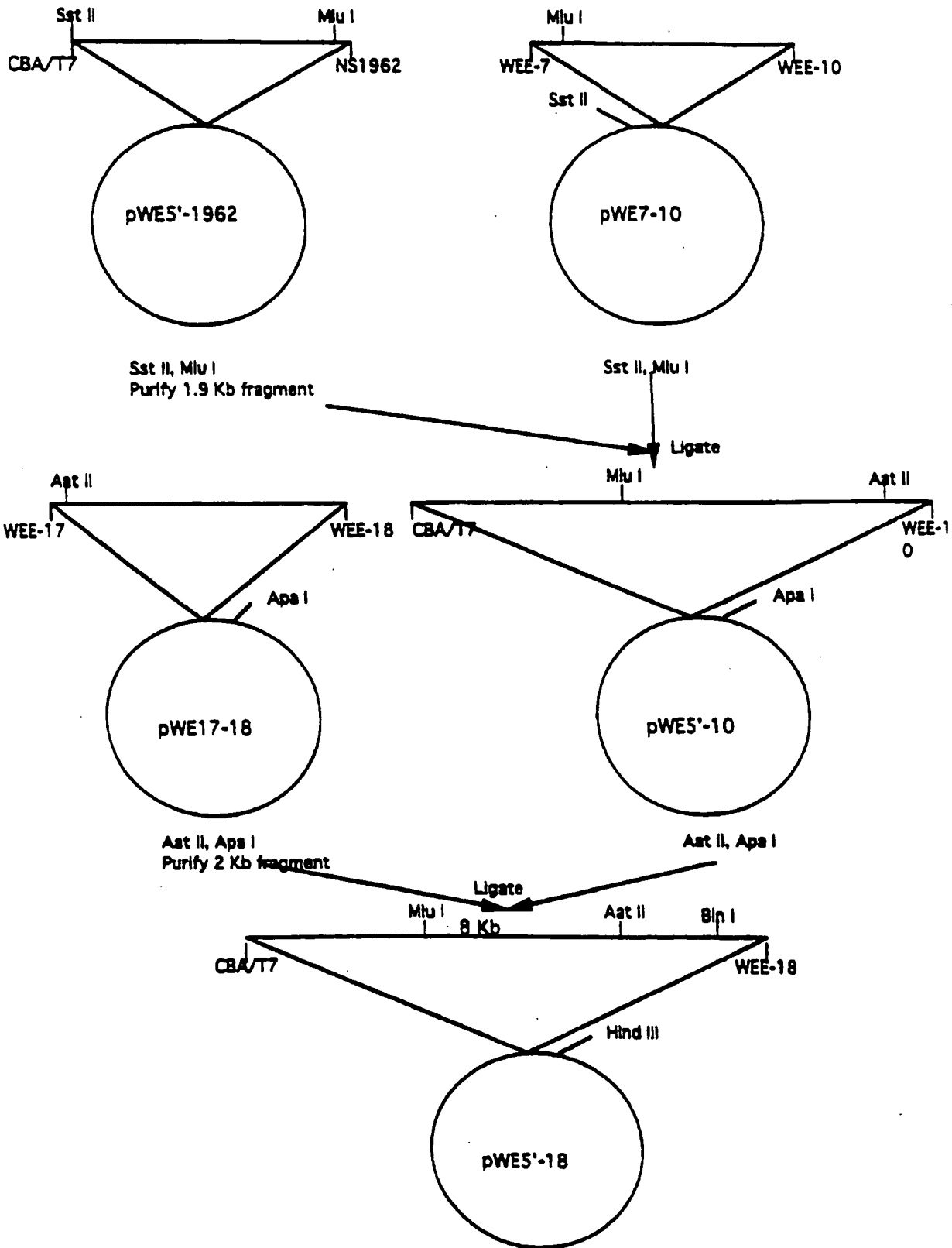


FIGURE 1B

Assembly of pWE2000 full length cDNA clone of western equine encephalitis virus from modules pWE3'-18 and pWE3'-17

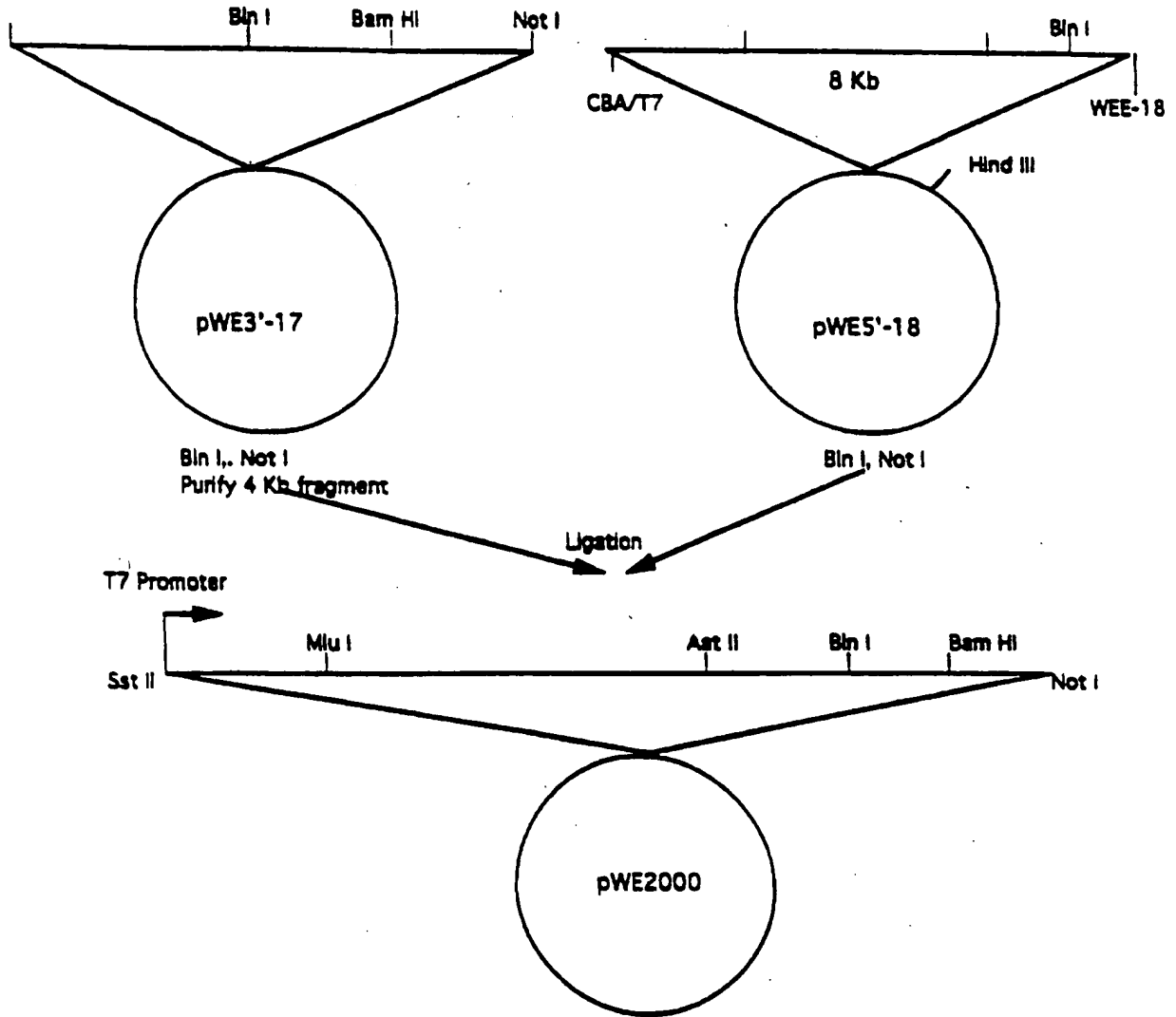


FIGURE 1C

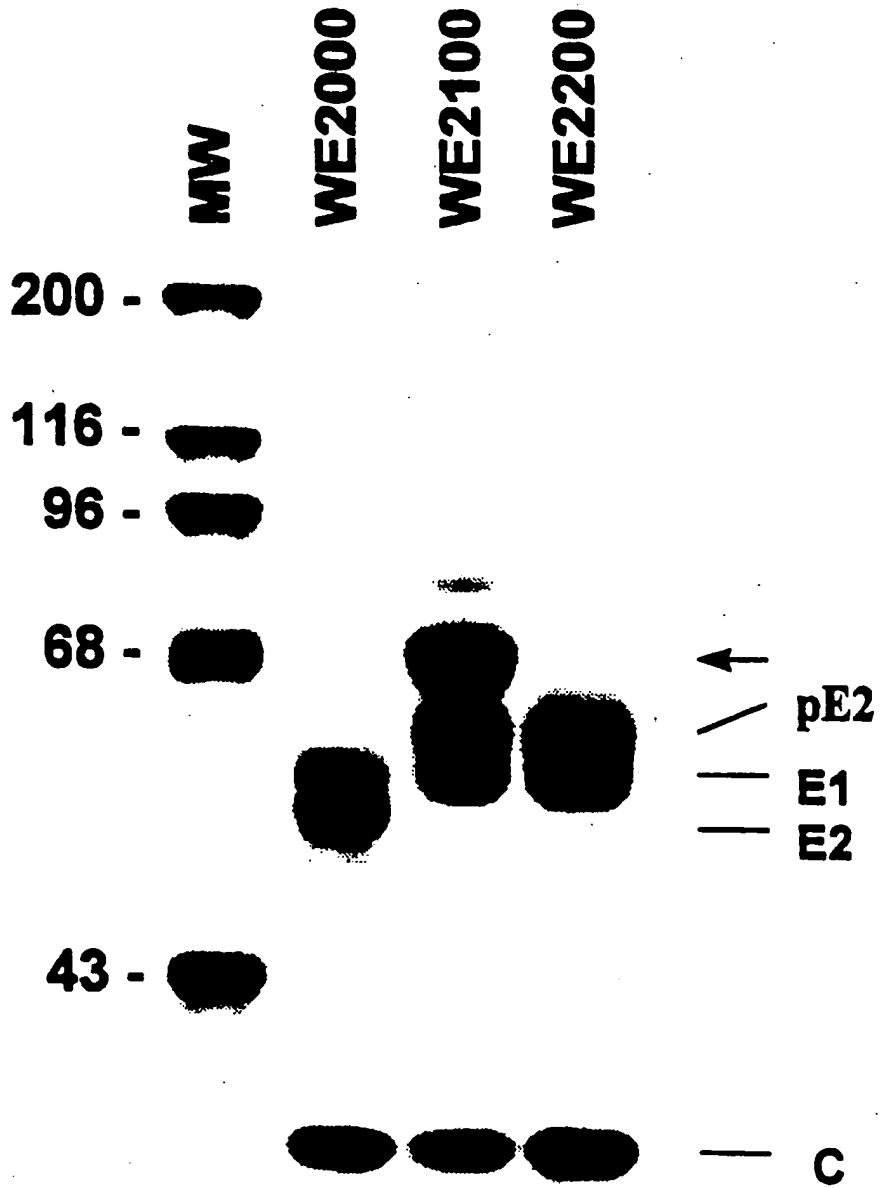


FIGURE 2

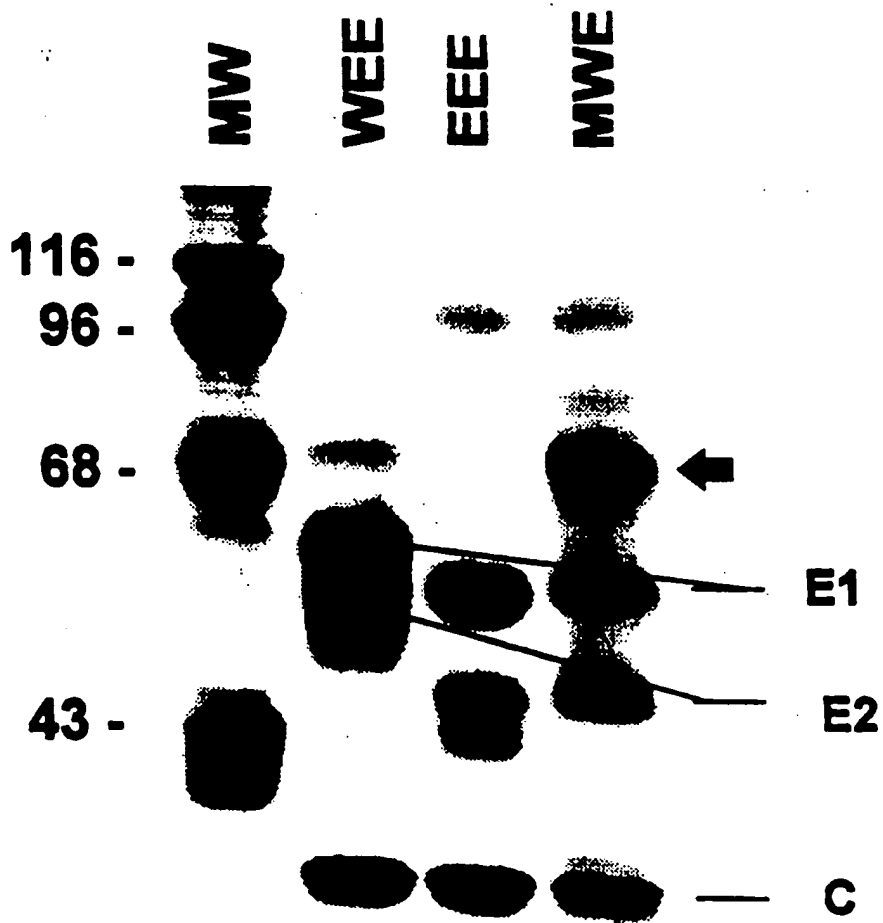
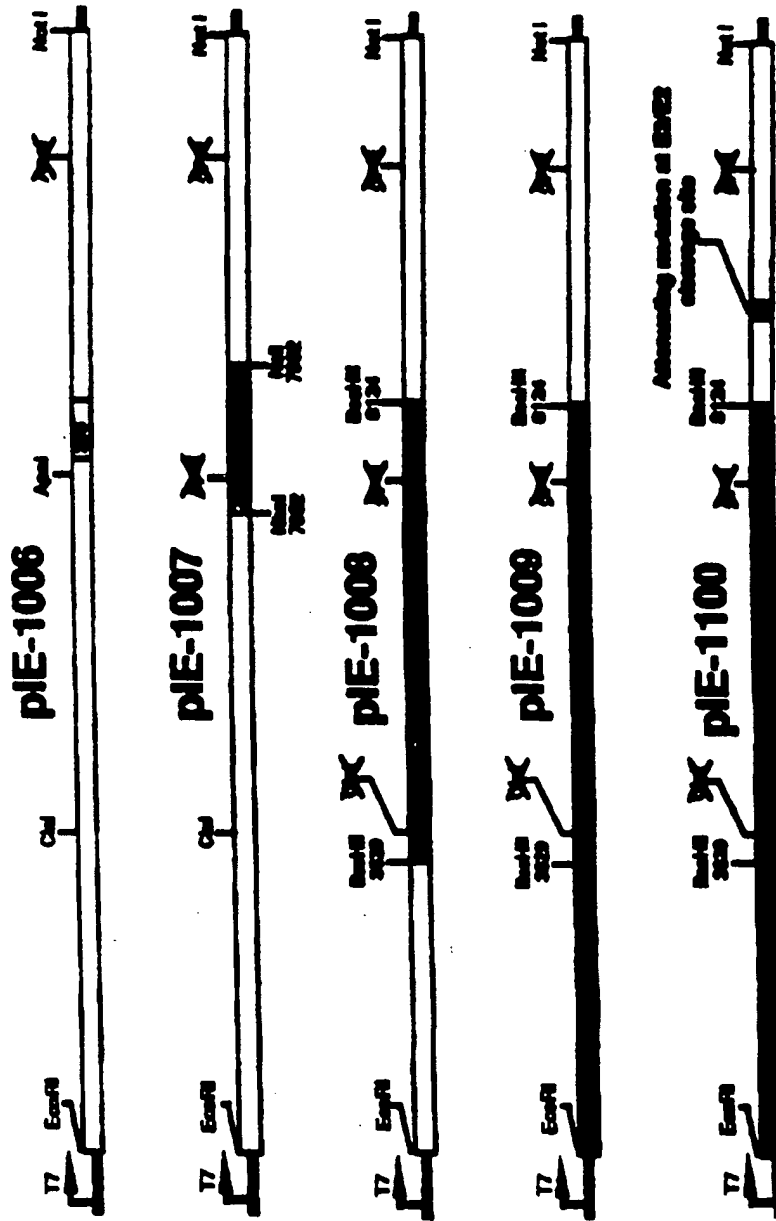


FIGURE 3

Figure 4



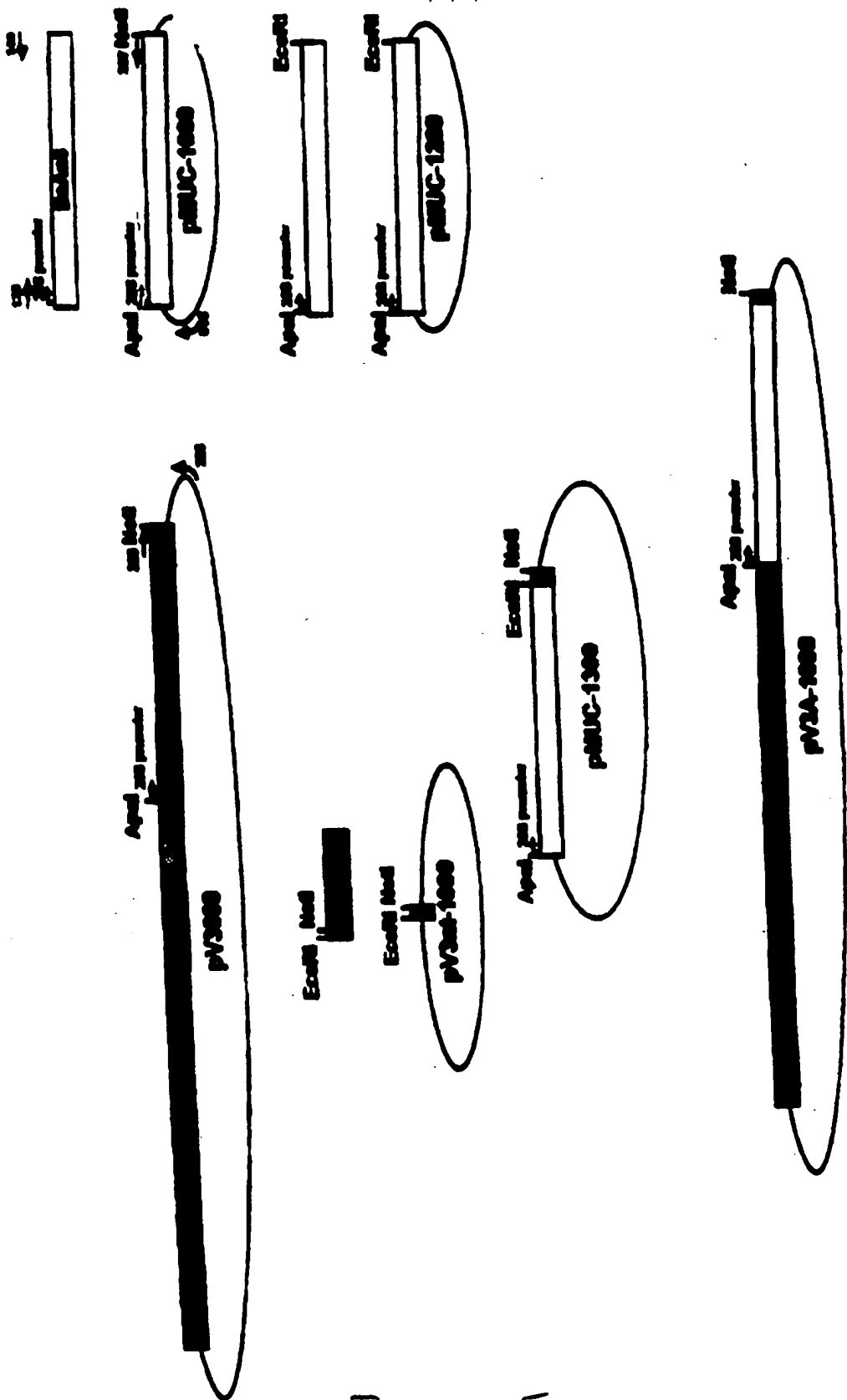


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10645

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(6) :C12N 15/40, 7/01, 7/04, 15/86; A61K 39/193.
 US CL :536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
 Minimum documentation searched (classification system followed by classification symbols)
 U.S. : 536/23.72; 435/235.1, 236, 69.1, 172.3; 424/205.1, 218.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 Aps, Biosis, Cab, Derwent WPI. Search terms: western, equine(w)enceph?, attenuat?, venezuelan, genom?, ma. clon?, sequenc?, dna, dnas, "E2", "E3", wee.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FRAIZER, G. et al. Isolation and preliminary characterization of mutants of Western equine encephalomyelitis virus with altered virulence in chickens. Biological Abstracts. 1985. Vol. 80, No. 5, page AB-506, Abstract no. 41179, see entire abstract.	16
Y	US 5,185,440 A (DAVIS et al) 09 February 1993, see entire document, particularly column 7, line 66 through column 8, line 1.	1-3, 8, 9, 16, 59
Y	US 5,505,947 A (JOHNSTON et al) 09 April 1996, see particularly column 3, lines 50-54, Abstract, and Examples 1 and 2.	3, 59

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 27 AUGUST 1998	Date of mailing of the international search report 06 OCT 1998
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer: <i>Mary E. Mosher</i> MARY E. MOSHER, PH.D. Telephone No. (703) 308-0196
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10645

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WEAVER, S.C. et al. A comparison of the Nucleotide Sequences of Eastern and Western Equine Encephalomyelitis Viruses with Those of Other Alphaviruses and Related RNA Viruses. <i>Virology</i> . 1993. Vol. 197, pages 375-390. See figures 3 and 4.	1-3, 8, 9, 16, 59
Y	HAHN, C.S. et al. Western equine encephalitis virus is a recombinant virus. <i>Proceedings of the National Academy of Sciences USA</i> . August 1988. Vol. 85, p. 5997-6001. See Figure 1.	1-3, 8, 9, 16, 59
A	STRAUSS, J.H. et al. The Alphaviruses: Gene Expression, Replication, and Evolution. <i>Microbiological Reviews</i> . September 1994. Vol. 58, No. 3, pages 491-562.	1-22, 37-40, 51-53, 59

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10645

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22, 37-40, 51-53, 59

- Remark on Protest The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/10645

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group 1, claims 1-22, 37-40, 51-53, 59, drawn to a first product DNA encoding an infectious genome of Western Equine Encephalitis (WEE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 2, claims 23-34, 41, 54, 59 drawn to second product DNA encoding an infectious genome of Venezuelan Equine Encephalitis IE variant (VEE IE) virus, RNA and virus encoded by DNA, vaccines and pharmaceuticals comprising virus encoded, and methods of use.

Group 3, claims 35-36, drawn to third product, combination vaccine comprising attenuated WEE and attenuated VEE IE

Group 4, claims 42-50, 55-58, drawn to fourth product, chimeric alphavirus and vaccine.

Group 5, claims 62, 68, drawn to fifth product, WEE specific primer kit, and method of use.

Group 6, claims 63, 69, drawn to sixth product, VEE IE specific primer kit, and method of use

Group 7, claims 70-72, drawn to seventh product, DNA encoding structural genes of VEE variant IIIA and products encoded.

Group 8, claims 60, drawn to method of diagnosing WEE using antigen.

Group 9, claims 61, drawn to method of diagnosing VEE IE using antigen.

Group 10, claims 64-67, drawn to method of vaccinating immune individual using a second alphavirus.

The inventions listed as Groups 1-10 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

In groups 1 and 2, the special technical feature in each case is the DNA encoding an infectious genome of a particular virus; since groups 1 and 2 pertain to two distinct viruses, they involve different special technical features.

Group 3 does not have the corresponding special technical feature, because the attenuated viruses required in group 3 are not limited to those made by using the DNAs of groups 1 or 2; for example claim 35 encompasses combination vaccines using viruses attenuated by propagation in culture.

Group 4 does not have the corresponding special technical feature, because it is not limited to either of the viruses encoded by the DNAs of group 1 or group 2; for example claim 42 encompasses a chimera made from Sindbis and Semliki Forest viruses.

Groups 5 and 6 do not have the corresponding special technical feature, because they require small, specific primers, not infectious full-length genomic materials.

Group 7 does not have the corresponding special technical feature, because is drawn to a sub-genomic fragment of a third virus.

Groups 8 and 9 do not have the corresponding special technical feature, because they are not limited to materials of groups 1 or 2; for example claim 60 encompasses a method using viral antigens produced by propagation of native WEE in standard tissue culture.

Group 10 does not have the corresponding special technical feature because it is not limited to the viruses made using the DNAs of groups 1 or 2; for example claim 64 encompasses a method of vaccinating a Sindbis-immune monkey

INTERNATIONAL SEARCH REPORT

International application No.
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against Semliki Forest virus.